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Emerging swine enteric coronaviruses: Comparison of pathogenicity and antibody response

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Emerging swine enteric coronaviruses: Comparison of pathogenicity and antibody response

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

I dedicate this thesis to my parents, Pat and Neil Gibson, who have always supported me and encouraged me to explore my curiosity from a young age. Also, I would like to dedicate this thesis to Kathy Long, my late aunt and godmother, whom is known for her hard work and early career achievements in science.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

Ab	Antibody
ASC	Antibody secreting cell
APC	Antigen presenting cell
ELISA	Enzyme-linked immunosorbent assay
HIV	Human Immunodeficiency Virus
IAV	Influenza A Virus
IFA	Immunofluorescence assay
PDCoV	Porcine deltacoronavirus
PEDV	Porcine epidemic diarrhea virus
PRCV	Porcine respiratory coronavirus
qPCR	Quantitative polymerase chain reaction
SECoV	Swine enteric coronaviruses
ss (+) RNA	Single-stranded positive-sense ribonucleic acid
SVN	Serum virus neutralization
TGEV	Transmissible gastroenteritis virus
vRNA	Viral ribonucleic acid

ABSTRACT

Porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) recently emerged in the U.S. swine population resulting in an estimated loss of $481 - $929 million U.S. dollars in 2013-14. Both PEDV and PDCoV cause diarrheic disease resulting in dehydration, weight loss, and sometimes death. Although pigs of all ages are susceptible to these viruses, suckling piglets suffer the most severe disease. During 2013, PEDV associated pre-weaning morality rates approached 100% in naïve breeding farms. Many studies to date have demonstrated the significant impact of PEDV and PDCoV infections in pre-weaning piglets, but information on the diseases impact on older animals is limited or absent. To address this issue, a comparative pathogenicity study was conducted to characterize PEDV and PDCoV infection using 6-week-old experimentally-infected nursery pigs. Animals were challenged and then periodically necropsied until 42 days post inoculation (dpi). As a result, both PEDV and PDCoV were pathogenic and caused diarrhea in growing pigs, although the duration of diarrhea was shorter than reported in pre-weaning piglets. Compared to PDCoV, PEDV caused more severe clinical disease including reduced average daily gain and higher viral load in tissues and feces. Additionally, PEDV caused lesions in small intestinal tissues, but no apparent lesions were observed in pigs inoculated with PDCoV. Differences in the magnitude and duration of serum antibody response was also apparent between PEDV- and PDCoV-infected animals when measured by indirect fluorescent antibody (IFA) test and serum-virus neutralization (SVN) assay. Unexpectedly, anti-PDCoV antibodies waned to undetectable levels by 28 dpi, while anti-PEDV antibodies were detectable through the end of the study (42 dpi). Taken together, the pathogenicity of PEDV and PDCoV in growing pigs clearly differed under the study conditions, warranting further studies to elucidate pathogenesis and immunobiology of these coronaviruses in different age groups.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

With the emergence of new swine enteric coronaviruses (SECoV), PEDV and PDCoV led to devastating consequences on U.S. swine production. PEDV and PDCoV cause similar disease as TGEV, a historical SECoV, but are antigenically distinct from each other. SECoV infect villous and sometimes crypt enterocytes inducing atrophic enteritis in young piglets which is manifested by clinical effusive diarrhea due to malabsorption and increases osmotic secretions. More specifically, PEDV and PDCoV are more virulent to neonate piglets than weaned pigs. For example, during the PEDV outbreak in 2013 up to 90% mortality was seen in naïve, pre-weaning piglets. In 2013-14, PDCoV had an estimated mortality rate ranging from 40% - 80% (Jung et al., 2016). While many pathogenicity studies for PEDV and PDCoV have been completed in neonatal and pre-weaning piglets, there is a lack of such studies in older animals. The studies presented in this thesis characterized comparative pathogenicity of SECoV in nursery pigs and evaluated the diagnostic performance of serological assays with experimentally derived serum.

Thesis Organization

This thesis is organized into three chapters. The first chapter presents a review of the literature including SECoV disease presentation, pathogenesis, and host immune responses to infection. The second chapter discusses specific research projects which compared SECoV pathogenesis and antibody response. The third, and final, chapter states general conclusions from the research chapters and provides future directions of SECoV research.

Literature Review

I. General overview of *Coronaviridae*

Taxonomy and virus family members

Coronaviruses (CoVs) are a diverse group of viruses belonging to the family *Coronoviridae*. In the order *Nidovirales*. Within the *Coronaviridae* family, there are four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. Alphacoronaviruses including human strains NL63 and 229E, porcine respiratory coronavirus, and feline coronavirus infect the epithelial cells of the lungs causing respiratory illness (Deng et al., 2016; Masters and Perlman, 2013). On the other hand, swine alphacoronaviruses, such as transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) in pigs, canine CoV, and bovine CoV infect the enterocytes lining the gastrointestinal track causing diarrhea (Kolb et al., 1998; Masters and Perlman, 2013).

Two viruses belonging to *Betacoronavirus*, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), cause severe respiratory symptoms and even death in human cases. In 2003, SARS-CoV emerged and caused an outbreak in humans attributing to 9% mortality in Asia and secondary cases globally. Himalayan palm civets were identified as the primary reservoir responsible for virus transmission to humans (Song et al., 2005). Other betacoronaviruses cause disease in domestic (murine) and livestock species. For example, bovine coronavirus and canine respiratory coronavirus cause respiratory illness, while porcine hemagglutinating encephalomyelitis virus (PHEV) causes a chronic wasting disease attributed to neurological symptoms (Masters and Perlman, 2013).

While alpha- and beta-CoVs are commonly detected in mammalian hosts, gammacoronaviruses and deltacoronaviruses are of primarily avian origin. The number of strains of avian CoVs is limited when compared to mammalian CoVs, including bat CoVs. With new advances in diagnostic technologies, more avian CoVs have been discovered. Due to the vast diversity in avian species susceptible to CoVs, it is suspected that avian-derived CoVs could be one of the primary reservoirs for emerging coronaviruses, like porcine deltacoronavirus (PDCoV) (Woo et al., 2012). Recently, Woo and colleagues identified three new avian coronaviruses: bulbul coronavirus HKU11, thrush coronavirus HKU12, and munia coronavirus HKU13. Collectively, PDCoV, a single porcine deltacoronavirus, and nine avian coronaviruses represent the genus *Deltacoronavirus*, while Beluga whale coronavirus SW1, avian infectious bronchitis virus, and turkey coronavirus make belong to *Gammacoronavirus* (Woo et al., 2012). The evolution of avian and mammalian CoVs were discussed in a recent publication. It was proposed that CoV diversity of avian species within *Gammacoronavirus* and *Detacoronavirus* and that of bat species in *Alphacoronavirus* and *Betacoronavirus*, but not vice versa, provided evidence for host specificity and virus evolution of CoV (Woo et al., 2012). In conclusion, both avian and mammalian CoVs diversity could attribute to its vast dissemination and transmission to other mammalian hosts, such as swine, although this still needs to be addressed.

Coronavirus structure and protein function

The single-stranded positive-sense RNA genome of *Coronaviridae* is the largest of all RNA viruses, measuring at 27-32 kilobases (kb) (Masters and Perlman, 2013). CoV are enveloped viruses with pleomorphic morphology and characteristic “crown-like” large peplomers protruding from the viral envelope, around 120 nm in diameter (Pensaert and De Bouck, 1978). There are four major structural proteins that make up the virion: spike (S),

membrane (M), envelope (E), and nucleocapsid (N). A subset of betacoronaviruses contain a fifth structural protein, hemagglutinin-esterase (HE), which binds sialic acid cell receptors on surface glycoproteins and assists the S protein in viral attachment through acetylesterase activity (Masters and Perlman, 2013). All the structural proteins except N are located in the viral envelope; N protein (55-58 kDa) is associated with the viral genome (Masters and Perlman, 2013).

As the largest structural protein ranging in size from 60 to 160 nm, S protein (180-220 kDa) forms a trimer complex the on the viral surface. S protein binds glycoprotein receptors on the surface of host cells which facilitates entry into the host cell. S protein includes 2 domains, S1 and S2, which is cleaved late in virion assembly allowing infectious virions to be released from the infected cell. S1 is extremely variable with low homology across genera while S2 is highly conserved across genera. Overall, S plays a key role in host tropism (Masters and Perlman, 2013). Based on the CoV genera and species, the S protein engages different host cell receptors. For instance, alphacoronaviruses bind through N-linked glycosylation to the host cell receptor, aminopeptidase N (APN) (de Groot-Mijnes et al., 2005; Deng et al., 2016; Li et al., 2016; Perlman and Netland, 2009; Saif et al., 2012). It has been shown in the presence of trypsin, that PEDV binds APN; however, it can also bind sialic acid, suggesting utilization of alternative receptors for viral attachment and entry *in vitro* (Kolb et al., 1998; Li et al., 2016; Taguchi et al., 2016). While similar, there are differences in the preferred receptors of emerging alphacoronaviruses.

In addition to S protein, some CoV, specifically betacoronaviruses, have an additional surface protein called the hemagglutininesterase (HE) protein. Specifically, murine coronavirus, betacoronavirus 1, and human CoV HKU1 have HE protein which can act with S protein to

facilitate cell attachment and entry (Masters and Perlman, 2013; Perlman and Netland, 2009). The main function of the HE protein is to bind sialic acid residues on the cell surface glycoproteins (Callebaut and Pensaert, 1980; King and Brian, 1982; Masters and Perlman, 2013). HE proteins assists S proteins in the attachment to host cells through HE protein's acetylesterase activity toward sialic acid residues on surface of the host cells (Klausegger et al., 1999; Masters and Perlman, 2013; Regl et al., 1999; Smits et al., 2005; Vlasak et al., 1988a, 1988b). Together, HE and S proteins bind host cells and facilitate viral entry.

M protein (29-36 kDa), the relatively conserved structural protein, is embedded in the virion envelope by three transmembrane domains which give the envelope its shape (Masters and Perlman, 2013; Saif et al., 2012). The M protein attaches to host cell's cytoplasm at the C-terminus. The N-terminus of M protein is cleaved by N-terminal signal peptides at the site of N-linked glycosylation in alphacoronaviruses. N-linked glycosylation by viral proteins may assist in virus-cell attachment, virus survival, virulence, and attenuation of the immune system. Alternatively, M protein O-linked glycosylation can occur in a subset of betacoronaviruses (Masters and Perlman, 2013). Specifically, glycosylation of host-cell membrane receptors by S and M proteins of SARS-CoV interacts with cell-associated lectins, specifically, LSECtin and DC-SIGN, which promotes viral attachment and entry (Vigerust and Shepherd, 2007). In TGEV, the M protein glycosylation site attributes to the viral tissue tropism and interferon (IFN) induction (Masters and Perlman, 2013; Saif et al., 2012). The N-terminus of TGEV M protein contains a single glycosylation site which was shown to induce Type I IFN *in vitro* (Baudoux et al., 1998). Additionally, M protein can bind TGEV complement-dependent neutralizing monoclonal antibodies (Godet et al., 1992; Saif et al., 2012; Woods et al., 1988). Due to high conservation of M protein among CoV, M protein stability may contribute to CoV host

specificity. However, these antigenic similarities are a major concern for cross-reactivity which may rule out CoV M protein's ability to differentiate antigens in serology.

E protein is a very small polypeptide structural protein, weighing only 8-12 kDa on average. E protein contains a short hydrophilic N terminus followed by a largely hydrophobic region and a large hydrophilic C-terminus tail. E protein is essential for functional virions. For example, when GFP-expressing infectious clones of TGEV were created with the E and M proteins knocked out. The E and M proteins were both shown to be essential for virion budding, as E and M protein defective constructs replicated, but could not produce progeny infectious virions. Additionally, a cloned TGEV replicon virus without a functional M protein proved lethal to the virion function, as no TGEV virus was recovered from infected cell cultures (Curtis et al., 2002; Masters & Perlman, 2013; Ortego et al., 2002). The E protein of CoVs insert into the ER, where they are transported with M and S to the site of virion assembly, the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). N protein in the ERGIC coalesce with E and S proteins to form virions. For other alpha-CoV like PEDV and PDCoV, E protein may also play a role in virion assembly, but their role in immunogenicity remains a mystery. PEDV E protein has been shown to have poor antigenicity *in vitro* which can be explained by its relatively small abundance compared with the other envelope proteins (Gimenez-Lirola et al., 2017). Additionally, E protein among different CoV can be widely divergent among CoV leading to a variety in how they interact with the immune system. Altogether, CoV E protein plays an essential role in virion assembly and has some immunogenic features, although they can vary based on CoV strain.

N protein is the only structural protein not housed in the viral envelope, where it's enclosed in the ribonucleoprotein core. N protein provides structural protection to the viral RNA

genome. The N-terminal domain of CoV N protein contains an RNA-binding groove where it binds viral RNA (vRNA) and forms a helical ribonucleoprotein complex (Fan et al., 2005; Grossoehme et al., 2009; Masters and Perlman, 2013; Saikatendu et al., 2007). CoV N protein undergoes a conformational change triggered by phosphorylation, enhancing its affinity for viral versus non-viral RNA (Chen et al., 2005; Masters and Perlman, 2013; Stohlman et al., 1988). N protein is known to be highly antigenic and relatively conserved. In such, closely related strains have potential to cross-react on serological assays. For example, PEDV and TGEV N proteins have been shown to cross-react in N protein-based ELISAs. However, truncation of the N-terminal region of the protein has shown to reduce such cross-reactivity in serological assays (Chen et al., 2016b; Okda et al., 2015).

Coronavirus genomic organization and gene expression

The CoV genome, ranging from 26 – 32 kilobases (kb), is the largest of all RNA viruses and contains seven to eight open reading frames (ORFs), of which the two largest ORFs overlap, along with the untranslated region at both 5' and 3' ends. Each gene is expressed via production of co 3'-end nested subgenomic mRNAs. The CoV genome encodes a large replicase gene which includes ORF1a and ORF1b, upstream from the viral structural protein genes (e.g., HE, S, E, M, N) and ORF3 (Masters and Perlman, 2013). The CoV replicase complex makes up almost two-thirds of the entire genome CoV and its product initiates the replication of viral genome and production of transcripts (i.e., mRNAs) for structural proteins and accessory proteins. (Masters and Perlman, 2013; ViralZone, 2017).

Coronavirus functional genomic studies have been done in murine hepatitis virus, a mammalian betacoronavirus, and TGEV due to the fact that a reverse genetic system (i.e. infectious cDNA clone) was available for these viruses. Upstream of all the structural protein

genes, making up a majority of the genome, is open reading frame 1ab (ORF1ab) which encodes replicase proteins. ORF1 encodes two polyproteins, ORF1a and -1b, from which 16 non-structural proteins (nsp) are transcribed. ORF1ab serves as the precursor responsible for viral genomic replication and transcription (Huang et al., 2008). A programmed -1 ribosomal frameshift of ORF1b is required for production of RNA-dependent RNA polymerases which initiate transcription of viral genomic proteins, including non-structural proteins (Perlman, 1998; Perlman and Netland, 2009; Plant and Dinman, 2008). A leader sequence at the 5' end serves as an anchor to produce subgenomic mRNAs (Perlman and Netland, 2009). The viral RNA-dependent RNA-polymerase (nsp12) uses a primase (nsp8) to produce the necessary primers which initiate viral RNA synthesis (Perlman and Netland, 2009). Additionally, nsps 7 – 10 act as co-factors to the other functional nsps, which play an essential role in subgenomic and genomic RNA replication (Deming et al., 2007).

PDCoV, a newly discovered deltacoronavirus, genomic organization is similar to other coronaviruses and is organized in the following manner: 5'- replicase ORF1ab, S, E, M, N - 3' (Masters and Perlman, 2013; Woo et al., 2012). PDCoV has the smallest genome of all mammalian coronaviruses measuring 26 kb which contains only 2 accessory genes, NS6 and NS7 (Woo et al., 2012). ORF NS6 is located between the M and N genes, while the other ORFs lie downstream from the N gene.

Coronavirus replication in vitro

CoVs contain protein domains functionally similar to other RNA viruses, such as: Human Immunodeficiency Virus and Influenza A Virus (IAV). The CoV S fusion peptide is exposed and interacts with the host cell membrane. A trimerization of the S protein monomers occur, resulting in the release of the CoV virion into the infected cell's cytoplasm (Masters and Perlman, 2013;

Saif et al., 2012). Clathrin-mediated endocytosis allows the virion into the cytoplasm moving along on actin filaments where nsps 7 – 16 assist in the formation of the replicase-transcriptase complex (RTC). Formation of the RTC precedes synthesis of the vRNA, the initial step in CoV replication. CoV replication occurs in double membrane vesicles derived from the cell's organelles called viral factories. Due to its single stranded positive-sense RNA genome, a double stranded RNA genome must first be synthesized (ViralZone, 2017). CoV transcription occurs through discontinuous extension of negative-stranded RNA synthesis, a template for the RTC (Masters and Perlman, 2013). CoV structural proteins (S, M, N, E) are inserted into the endoplasmic reticulum (ER). From the ER, they transit to the site of virion assembly, the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). CoV N protein in the ERGIC associates with the other structural proteins in the envelope to form virions. Complete virions are released from the ERGIC in vesicles, then travel along actin filaments, through the cytoplasm, and finally release from the cell through exocytosis.

In addition to the essential structural protein genes, CoV genome contains accessory genes which are not required for virus growth or expressed by all coronaviruses. Accessory genes can regulate and coordinate viral gene expression and function, along with some other ancillary roles. These accessory protein genes are located in the region between S and E protein genes as well as 3' to the N protein gene of TGEV, PRCV, and PEDV (Huang et al., 2008). In TGEV, the region between S and E proteins genes contains the accessory genes ORF3a and 3b. While not critical for TGEV replication, ORF3b has been shown to produce glycosylated M protein *in vitro* (Huang et al., 2008). Other SECoV genomes contain homologs of the ORF3a and 3b genes. Additionally, accessory gene 7, located at the 3' end of N, is present in TGEV but not in other SECoV, where it encodes a hydrophobic protein of 9 kDa. This accessory protein does attenuate

the virulence of virus if deleted as shown *in vivo*, but does not affect virus replication *in vitro* (Huang et al., 2008; Ortego et al., 2003). While SECoV accessory proteins are common, they do not play an essential role in virion function, but can interfere with virion processes as evidenced with TGEV.

II. Swine coronaviruses

Taxonomy and history

Swine CoVs are classified into 3 genera: *Alphacoronavirus*, *Betacoronavirus*, and *Deltacoronavirus*. Within the *Alphacoronavirus* genus, there are several swine coronaviruses. The two swine CoV that cause enteric disease, include TGEV and PEDV. Another alphacoronavirus, PRCV, is a deletion mutant of TGEV and a swine respiratory pathogen. Porcine hemagglutinating encephalomyelitis virus (PHEV), a betacoronavirus, causes a neurologic disease. PDCoV is the only porcine deltacoronavirus which causes enteric disease similar to TGE and PED. Collectively, TGEV, PEDV, and PDCoV have been proposed to be swine enteric coronaviruses (SECoV); a collective group of CoVs that are able to cause diarrheic disease in pigs of all ages, although disease is most severe in suckling piglets (Curry et al., 2017; Saif et al., 2012; Saif, 1996).

In 1946, TGEV, an emerging pathogen which caused a severe malabsorptive diarrhea, was first isolated (Doyle and Hutchings, 1946). The virus became endemic in the U.S. swine population, continuing to cause significant pre-weaning mortality and diarrheic disease in pigs of all ages. In 1984, a TGEV mutant known as PRCV later emerged (Laude et al., 1993). PRCV contains a substantial deletion in the S gene of TGEV, changing its cell tropism to cause a respiratory disease instead of enteric disease. Porcine respiratory coronavirus replicates in the nasal mucosa, trachea, and lungs of pigs causing mostly a mild or subclinical infection (Saif,

1993). Due to their antigenic similarities, it was observed that PRCV-exposed dams can partially protect their nursing piglets from TGEV (Bernard et al., 1989; Lanza et al., 1995; Paton & Brown, 1990). Today, PRCV antibodies are detectable in the serum and colostrum of most lactating sows and prevalence of disease caused by PRCV and TGEV is not common.

In Europe, a new alphacoronavirus, PEDV was discovered in 1971 and caused diarrheic disease similar to TGE, but was antigenically different from TGEV (Pensaert & De Bouck, 1978; Saif & Wesley, 1992; Wesley et al., 1997). A common historical strain of prototype PEDV (now commonly referred to as G1), CV777, was endemic in multiple countries across Europe and Asia (Alvarez et al., 2016; Choudhury et al., 2016; Lin et al., 2016). In 2010, a new strain of PEDV called variant PEDV (now commonly referred to as G2) caused devastating mortality in pre-weaning pigs across Asia. Shortly after, the similar variant PEDV was identified in the U.S. in 2013 (Stevenson et al., 2013). Variant PEDV emerged in the U.S. with each of the strain's lineage highly related to the 2012 variant PEDV strain identified in Anhui Province in China, although it is unclear how the first index case of PED arose in the U.S. (Huang et al., 2013). Beginning in 2013, the PEDV U.S. prototype strain quickly spread across North America causing epidemics of diarrhea and mortality in 90% of pre-weaning piglets in the United States, Canada, and Mexico (Chen et al., 2014; Jung et al., 2015; Madson et al., 2014; Marthaler et al., 2014).

Porcine epidemic diarrhea virus is highly infectious and spreads through ingestion or inhalation of virus-contaminated feces from infected pigs (Stevenson et al., 2013). In addition, PEDV can be transmitted by contact with fecal or organic matter containing infectious PEDV through improperly decontaminated animal trailers and farm equipment, known as fomites (Lowe et al., 2014). Other studies have observed that proximity of uninfected swine herds to

PEDV-infected herds could have played a factor in its rapid dissemination in the U.S. (Alvarez et al., 2016; Bottoms et al., 2013; Pensaert & Martelli, 2016). Finally, feedstuff contaminated by PEDV is a potential risk to disease spread; however, the ability for contaminated feedstuffs to cause disease has not proven successful under experimental conditions (Opriessnig et al., 2014; L. Wang et al., 2016).

Starting in 2014, PED outbreaks continued to occur, but were clinically less severe than observed with PEDV U.S. prototype strains. Upon sequence analysis, PEDV with deletions and insertions in the S gene was identified and later referred to as PEDV S-INDEL (also known as PEDV S gene variant). The PEDV S-INDEL strain demonstrated a less severe clinical disease presentation than PEDV U.S. prototype strain, which has been observed under experimental conditions. (Chen et al., 2016; Lin et al., 2015).

PDCoV was detected in diagnostic samples from U.S. swine with diarrhea as early as 2013 through a retrospective analysis, although it wasn't reported until 2014 (Sinha et al., 2015; Wang et al., 2014; Zhang et al., 2016). PDCoV caused an estimated 40% mortality rates of infected suckling piglets, which is less than PEDV infection that can approach greater than 90% (Jung et al., 2016; Schulz and Tonsor, 2015). During the 2013-2014 outbreak, the emergence of PEDV and to a lesser extent PDCoV cost the U.S. economy anywhere from $900 million to $1.8 billion when reduced annual pig loss, loss on hog returns, and decreased annual consumer surplus are taken into consideration (Paarlberg, 2014; Schulz and Tonsor, 2015).

Pathogenesis and pathogenicity

SECoV infect enterocytes of the small intestine causing effusive diarrhea and/or vomiting which is indistinguishable among SECoV. Malabsorptive diarrhea caused by SECoV infection leads to a reduced absorptive capacity of the intestinal villi due to malabsorption and increased

osmotic secretions (Chen et al., 2015; Madson et al., 2014). Additionally, the severity of clinical diarrhea can lead to dehydration and eventually mortality in naïve neonatal piglets. While clinical disease is similar among the emerging CoVs, PEDV and PDCoV, there are some differences when it comes to the disease impact on young animals. More specifically, PEDV-infected animals suffer lethargy and appetite loss causing reduced daily feed intake during the acute clinical phase (Curry et al., 2017). On the contrary, PDCoV-infected animals become lethargic, but have a normal feed intake. Due to the malabsorption and reduced intake of feed caused by PEDV infection, nutrients are lost resulting in poor performance by the animal (Curry et al., 2017, 2016).

The severity of clinical disease in pigs infected with PEDV cam vary which may relate to a difference in pathogenicity by strain. For example, classical PEDV, such as CV777 strain, and prototype PEDV strains cause similar acute gastroenteritis and intestinal lesions as the virulent TGEV Miller strain (Callebaut et al., 1982; Pensaert, 1992; Pensaert and Martelli, 2016). Other studies found similar finding in 3-day old and 1-week old piglets infected with other virulent prototype PEDV strains (Lin et al., 2015; Yamamoto et al., 2015). PEDV infection can cause prolonged viral detection in young piglets. PED vRNA detection in the feces of pre-weaning piglets and exposed dams extended to 14 – 35 dpi, despite the resolution of clinical diarrhea by 7 – 10 dpi (Vitosh-Sillman et al., 2016). On the other hand, after the emergence of PEDV in the U.S., a new variant PEDV strain named PEDV S-INDEL was detected (Lin et al., 2015; Oka et al., 2014; Yamamoto et al., 2015). As compared to PEDV U.S. prototype strains, PEDV S-INDEL infected piglets resulted in diminished clinical signs of disease including lower histopathological lesion scores (i.e. less severe lesions) in intestinal tissues and lower viral genomic copies in feces and when compared with the U.S. prototype PEDV infected piglets

(Chen et al., 2016), suggesting that PEDV S-INDEL strains are less virulent than prototype PEDV strains.

Differences in the viral load detected in the intestinal tissues and feces of different aged pigs infected with highly virulent PEDV strains have been demonstrated. Moreover, age-dependent pathogenicity to the PEDV U.S. prototype infection has been studied in pre-weaning and post-weaning piglets, with limited research done on growing pigs. In PEDV-infected neonates, slower turnover of infected enterocytes and lack of immunological memory play a role in severe pathogenicity. For example, infected nine-day-old pigs had significantly higher viral titer in feces than the 26-day-old pigs during the clinical phase of disease (Jung et al., 2015). When comparing clinical disease between 9-day-old and 21-day-old piglets, the older piglets developed diarrhea starting at 2 – 3 dpi, while the neonatal piglets suffered from severe watery diarrhea starting at 1 dpi. While the age-dependent mechanism is unclear, research has shown pathogenicity differences in the intestinal function of pre-weaning versus weaned pigs in response to PEDV. In PEDV U.S. prototype infected neonates, impaired crypt stem cell and lytic activity of infected enterocytes, disrupted intestinal barrier with shortened villi and widening of intestinal crypts, and reduced numbers of interferon-gamma (IFN-γ) producing cytotoxic cells (NK cells) (Annamalai et al., 2015; Curry et al., 2017, 2016; Jung et al., 2015). Taken together, PEDV causes comparable diarrheic disease, but the age of the pig can play a role in PEDV pathogenicity.

In PDCoV pathogenicity studies, the most severe disease occurs in suckling piglets, although post-weaning piglets are susceptible to infection, but to a lesser extent than observed with PEDV. Similar to PEDV and TGEV, PDCoV induces acute effusive diarrhea, sometimes associated with vomiting (Jung et al., 2016). PDCoV infected enterocytes of pre-weaning piglets

undergo villous atrophy in the small, but not the large intestine; the majority of small intestinal villous enterocytes are infected, with very few crypt cells being infected (Jung et al., 2016). Additionally, an experiment using four-day-old pigs showed some non-intestinal tissues positive for virus by PCR, such as the lung and mesenteric lymph node, although at low levels (Vitosh-Sillman et al., 2016). PDCoV infection in 5-day-old and 14-day-old piglets developed watery diarrhea and shed virus in their feces at highest RNA levels from 3 – 5 dpi, although duration of vRNA detection varied from 7 – 21 dpi between the different aged groups (Chen et al., 2015; Hu et al., 2016). There can also be differences between PEDV and PDCoV infection in pre-weaning piglets. While clinically both infections present similar manifestation, there can be differences in the degree of pathogenesis caused in pre-weaning piglets. For example, 5-day-old PDCoV-infected piglets developed severe atrophic enteritis, however, lesions were only observed in the jejunum and ileum with peak lesions at 3 – 4 dpi (Chen et al., 2015; Hu et al., 2016). In contrast, PEDV infection in 5-day-old piglets caused more severe and diffuse lesions throughout the small intestine, with virus detected throughout the small and large intestine (Thomas et al., 2015). The duration of shedding in PEDV infection is also greater than with PDCoV. Although studies have not assessed fecal shedding duration in neonates, 3-week-old weaned pigs infected with PEDV continued to shed PEDV in feces until 24 dpi (Madson et al., 2014).

III. Host Immune Response to CoV Infection

Porcine mucosal immunity

The mucosal barrier of the gastrointestinal tract is made up of epithelial cells lined with mucus; with the help of secretory IgA (sIgA), together they establish a protective barrier which keeps potentially harmful antigens within the lumen where they can be removed. sIgA antibodies play a major role in preserving the intestinal barrier by blocking or neutralizing invading

pathogens (Murphy et al., 2012; Saif, 1996; Stokes et al., 1991; Yuan and Saif, 2002). Dimeric IgA can bind antigens on the basolateral surface of the epithelium and transport them across the epithelial cells via PIgR, where they can be dumped into the intestinal lumen and degraded (Saif, 1996; Yuan and Saif, 2002). The dynamic function of the mucosal immune system not only prevents pathogens from invading the epithelium, but also may help to mitigate and clear infections when foreign antigens have penetrated through. In order to maintain this balance, the majority of intraepithelial lymphocytes (IEL) express CD2 which regulates their activation and suppression (Camerini et al., 1993). In older animals, CD8+ T cells interact with intraepithelial immune cells to secrete IL-2 and IFN upon antigen stimulation which have some cytotoxic capabilities (Saif, 1996; Stokes et al., 1991). Other components of the mucosal immune system are classified as mucosal associated lymphoid tissue (MALT). MALT are immunological structures that generate different responses to pathogens, without causing harm to commensal microorganisms. MALT is separated into two categories: 1. Lymphoid structures, including: Peyer's patches (PP) and mesenteric lymph nodes (MLN) and 2. Diffuse lymphoid tissue, including the lamina propria (LP).

Peyer's patches are found in the jejunum and ileum of the small intestines which contain multiple B cell follicles separated by intrafollicular areas where T cells reside. M cells exist between the epithelium and the follicle of the PP where antigen-presenting cells are present; more specifically, the presence of Ig-secreting plasma cells, dendritic cells, and intraepithelial lymphocytes. M cells play an important role in initiating both innate and adaptive immune responses through transepithelial transportation of antigen from the lumen to lymphocytes (Corr et al., 2007; Gebert et al., 1996; Murphy et al., 2012; Stokes et al., 1991; Turner, 2009). Differentiated lymphocytes migrate from the PP where it enters the lymphatics and migrates to

other lymphoid tissues, such as the MLN (Saif, 1996). The MLN is rich in T and B lymphocytes which are activated upon by antigen presentation and activation from APCs, mainly dendritic cells (DCs) that drain from the intestine and migrate to the MLN through the afferent lymphatics (Macpherson and Smith, 2006; Moretó and Pérez-Bosque, 2014). A recent study has shown the MLN as the primary site for immune tolerance to food antigens and commensal gastrointestinal microbes (Worbs et al., 2006). Additionally, the MLN acts as a border that mitigates transportation of immune cells and antigen between the mucosal immunity and systemic immune system (Macpherson and Smith, 2006).

Next, the LP contains immune cells, including macrophages, dendritic cells, neutrophils, lymphocytes, and plasma cells, which are activated in response to an antigen. The LP is the layer underneath the basement membrane and is an important component of induction and regulation of the gastrointestinal barrier (Murphy et al., 2012; Turner, 2009). The LP is in direct contact with the epithelium which plays an important role in the maintaining homeostasis. However, the immune cells within the LP become activated and induce local cell-mediated immunity (CMI) in context of disease-causing dysbiosis (Murphy et al., 2012; Turner, 2009). The overall function of LP lymphocytes prevents expression of active T cell responses to antigens normally present in the intestinal lumen which maintains immunological tolerance to self-antigen (Murphy et al., 2012; Saif, 1996).

Maintaining integrity of the mucosal barrier is key to preventing infection. When the barrier's integrity is disrupted, a state called dysbiosis, then other immune defenses are initiated which cause a pro-inflammatory state. The ability to regulate the barrier lies with the mucosal immune components which were discussed previously. The porcine gastrointestinal immune

system of young pigs are slow to mature and develop, increasing their susceptibility to disease when compared with that of adult pigs (Stokes et al., 1991).

At weaning, pigs undergo changes in the gastrointestinal barrier in response to stress (i.e. changes in diet, transportation, and/or environment), increasing their susceptibility to enteric infections. The disruption of the barrier in conjunction with the immaturity of the young pig's mucosal immune system provides an opportunity for pathogens to invade (Saif, 1996; Stokes et al., 1991). Stress caused by weaning along with immaturity of mucosal immune system leads to disruption of the mucosal barrier which leaves the young pig susceptible to enteric infection.

Mucosal immune responses to swine viral infections

Mucosal and systemic immunological responses to enteric viral infections vary in location of immune induction and regulation, the virus phenotype, and duration of immunity. All of these factors impact immunological memory and protection from re-infection. Immunological memory responses to enteric viral infection can be separated into short-term effector memory and long-term memory responses (Yuan and Saif, 2002). A human rotavirus (HRV) challenge model was used to describe the immune activation mechanism of effector and memory antibody secreting cells (ASC) locally and systemically during a primary and homologous oral challenge (Yuan et al., 2001). In this study, gnotobiotic pigs were challenged orally with 1 dose of a virulent HRV or 3 doses of an attenuated HRV and subsequently challenged with virulent HRV and observed for intestinal and serum ASC. The study showed that after challenge, pigs inoculated with the virulent strain elicited higher numbers of IgA ASC and memory B cells in the ileal LP and MLN than pigs inoculated with the attenuated strain which consequentially elicited higher systemic IgG ASC and memory B cells in the spleen than the induction site (Yuan et al., 2001). This short-term effector and memory response at the MALT begins to wane in the weeks following disease recovery most

likely due to development of protective mucosal immunity as other previous studies have reported that IgA mucosal effector immune responses are correlates of protection against swine enteric viral infections (Saif, 1996; Yuan et al., 2001; Yuan and Saif, 2002). In contrast, systemic IgG memory B cells remain at the peak level in the pig's spleen and contribute to long-term memory, although it is not shown to be protective (Yuan et al., 2001). Based on this information, direct mucosal priming with live virus induces an effective and protective memory response, but requires boosting with antigen periodically to maintain protective levels against swine enteric viral infection.

Furthermore, both immunological and physiological changes occur in the small intestine due to this disruption. Increased permeability across the gastrointestinal barrier associated with effusive viral diarrhea dysregulates the absorptive capacity and nutrient uptake in the small intestine, leaving the animal malnourished. In suckling piglets, this malnourishment can lead to death. Also, local immune responses are impacted by changes in the barrier. A naïve mature pig relies on a robust innate immune response to help clear SECoV infections. As suckling and immature piglets are the most susceptible to infection due to the lack of immunological development and memory, proper maternal immunity is required in absence of a robust innate immune response (Stokes et al., 1991). As the pig matures and develops immune memory, a robust immune response can be activated and help clear infection. When comparing a suckling piglet's immune response to a weaned pig's immune response to PEDV infection, the neonate pigs had lower NK cell frequencies and low IFN-γ producing NK cell populations compared with weaned pigs, which may explain more severe clinical disease in the suckling piglets compared to the weaned pigs (Annamalai et al., 2015). In the same study, a higher abundance of CD4+ T cells were observed in the ileum of suckling pigs compared to weaned pigs; however, no differences were observed in the CD8+ T cells frequency and the functionality of these cell

populations in neonates was not discussed (Annamalai et al., 2015). Collectively, neonate pigs elicit more severe disease manifestation when compared to weaned pigs which may be due to the absence of a vigorous immune response.

Structural and physiological changes to the intestinal tract of pre-weaning piglets are observed during viral enteric infections. One study evaluated the local innate immune response during an enteric infection which reported increased pro-inflammatory cytokines secreted at the site of infection, along with malabsorptive diarrhea in pigs exposed to PEDV (Annamalai et al., 2015). Additionally, barrier disfunction was attributed to increased paracellular uptake accompanied by irregular distribution and reduced expression of tight junction proteins and intestinal goblet cells in the small intestine of PEDV-infected pigs (Curry et al., 2016; Jung, Kwonil; Saif, 2016; Pearce et al., 2016). Another example of this was published where PEDV infection of 9-day-old pigs caused morphological changes, including: irregular distribution and reduced expression of tight junctions and adherens junction proteins in small intestinal villi (Jung et al., 2015). Additionally, PEDV infection of 3-week-old weaned pigs altered several intestinal proteins involved with cell migration, proliferation, differentiation, apoptosis, and structure, as well as immunological response to infection (Pearce et al., 2016). Finally, PEDV can induce dysbiosis of the gastrointestinal barrier by increasing populations of obligate anaerobic, non-spore forming bacteria during infection; this dysbiosis can lead to changes in the gut health and metabolism (Koh et al., 2015). Overall, decreased immunity during times of stress along with underdeveloped mucosal immune systems in younger pigs can lead to susceptibility to enteric viral infections, such as PEDV.

Lactogenic immunity to SECoV

Piglets are born agammaglobulinemic due to an impermeable placenta accompanied by a weakened mucosal immunity and require the transfer of maternally derived immunity for protection from pathogens to which they may be exposed to postpartum. Commonly referred to as lactogenic immunity, immunoglobulins (Ig) and some APCs, although these APCs are short-lived, are passively transferred to the suckling piglet through ingestion of the dam's colostrum and milk. Of the Ig that are present in mammary secretions, IgG and IgA are predominant in colostrum and milk. IgG is present at a high level in colostrum during the first 24 – 48 hours post-partum. As lactation progresses, IgA becomes the dominant Ig in the dam's milk and remains at a high level due to its stability. Studies have shown, 80% of IgA is located in GALT and it is the primary Ig involved in conferring lactogenic immunity to suckling piglets against enteric pathogens (Lanza et al., 1995). Oral vaccination of live, but not attenuated, TGEV has shown to induce a strong sIgA response in the mammary gland of pregnant and post-partum sows. This mechanism is activated when TGEV IgA plasmablasts in the gastrointestinal tract stimulated via oral vaccination or virulent pathogen and migrate to the LP and mammary gland via the network of lymphatics (Bohl et al., 1972; Saif et al., 1972; Song et al., 2015). The ingested passively acquired IgA has been shown to be protective (0% mortality) in piglets against experimental TGEV and PEDV challenges via oral route (Bohl et al., 1972; Lanza et al., 1995; Saif, 1993; Saif et al., 1972). In contrast, sow vaccination with inactivated TGEV Ag induced IgG predominantly, which rapidly decreases in milk 7 days post challenge and hence provided little lactogenic immunity to piglets (Lanza et al., 1995). The development of maternal vaccination strategies, such a live vaccination, which induce mucosal immunity, and therefore

sIgA in the milk, have been proven successful in the case of TGEV, and potential to protect piglets from other SECoV.

Immune cells migrate to the mammary gland through homing in and out of the secondary lymphoid organs by crossing the vessels' high endothelial venules (HEV); B and T lymphocytes extravasate through HEV (Murphy et al., 2012). During parturition, IgA and T lymphocytes in the intestine traffic to the mammary gland and are expressed in the sow's colostrum. Mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) expression on blood vessel endothelial cells in the mammary gland increase post-partum into early lactation (Bourges et al., 2008). Additionally, expression of mammary gland homing receptors, chemokine receptor 10 (CCR10) and chemokine ligand 28 (CCL28), increase in the mammary gland, resulting in peak IgA-secreting B cells at the time of farrowing (Langel et al., 2016). Throughout lactation, homing receptor CCL28 is continually expressed to encourage lymphocyte homing to the mammary gland in pigs (Bourges et al., 2008; Langel et al., 2016). When the homing mechanism is activated through antigen presentation in the GALT, Ig-secreting lymphocytes traffic to the mammary gland where sIgA is secreted in colostrum and milk conferring protective immunity.

Physiochemical changes influenced by progesterone and estrogen levels during a sow's pregnancy can promote the selective transport of IgG from the serum to the mammary gland, where IgG is secreted in the colostrum. However, IgG can be degraded by the piglet's small intestine starting one week post-partum, but can persist longer in circulation. In contrast, sIgA is resistant to proteolysis in the small intestine and can persist in the intestines longer than IgG, which is important for mucosal immune protection. During the first week of life, piglets can compensate for degradation of intestinal antibodies through physical changes in the gut's integrity. For example, 1 – 7 day-old-piglets can absorb and transport passively-acquired

maternal lymphocytes across the small intestinal epithelium, where they can induce local immune responses (Tuboly et al., 1988). From previous studies, PEDV-specific IgG is passively transferred through colostrum to suckling piglets for the first few days post-partum, but PEDV-specific IgA is continually transferred to suckling piglets by milk throughout lactation, essential for suckling piglet protection from PEDV (Goede et al., 2015; Lanza et al., 1995).

While lactogenic immunity has been well characterized with TGEV maternal vaccination, there is limited evidence on PEDV or PDCoV infections. As with TGEV, sows exposed to infectious PEDV relayed partial protection to suckling piglets against virulent PEDV challenge (Goede et al., 2015). While increased IgG levels were transferred to suckling piglets from sows administered a killed PEDV vaccine versus naïve sows, the IgG levels did reduce mortality of piglets exposed to live PEDV (Schwartz et al., 2016). These results suggest killed vaccination of sows do not induce sufficient passive immunity in the form of IgA when compared to sow exposure to live virus. Furthermore, sufficient passive immunity of IgA is required to protect piglets from pre-weaning mortality.

Prophylaxis treatment is another option for protecting susceptible neonates, however, this approach is not well studied for PEDV or PDCoV. One study evaluated the effects of PEDV challenge on suckling piglets that were administered with different levels of PEDV-specific antibodies (primarily IgG) via intraperitoneal injection before being exposed to PEDV. This study showed piglets that received the passively acquired systemic antibodies had increased survival and returned to normal body temperature sooner than the untreated challenged piglets independent of dose (Poonsuk et al., 2016). However, treated piglets still suffered from severe malabsorptive diarrhea and some mortality, suggesting prophylaxis treatment of piglets with PEDV-specific antibodies ameliorate, but do not fully protect against PEDV.

IV. Diagnostics of SECoV

Various diagnostics are available for PEDV and other SECoV. Virological methods target the detection of viral proteins, vRNA, or the virus itself, while serological methods detect virus-specific antibodies. Generally, diagnostic samples including serum, feces, tissues, oral fluids and milk are collected from infected animals. If the animals suffered mortality, the animal is necropsied and samples are taken, such as intestinal tissues and intestinal content, are taken for SECoV laboratory testing. For herd monitoring, samples from live animals including oral swabs, serum, and feces are taken. Since the 2013 PEDV outbreak, new diagnostic tools have emerged that have led to the rapid diagnosis of SECoV.

Virological assays

There are two categories of virological tests a) tests for the detection of viable virus and viral antigen and b) detection of vRNA. Virus isolation (VI), tissue immunoassays such as frozen tissues section immunofluorescence (FTIF) and immunohistochemistry (IHC), and antigen-capturing ELISA are all examples of tests that detect viable virus or viral antigen, while polymerase chain reaction (PCR) tests detect the vRNA of SECoV. VI involves infecting susceptible cells with suspect clinical specimens, generally feces or intestinal content, and allowing the virus to infect and grow in cell culture. VI is used to detect or identify the target virus; however, VI can be a time-consuming and laborious process with a low success rate due to its low diagnostic sensitivity and potential cell culture toxicity from clinical specimen (Chen et al., 2014; Jung and Hu, 2016; Oka et al., 2014; Shibata et al., 2000). FTIF and IHC both detect antigens by staining with antibodies (Callebaut et al., 1982; Jung et al., 2015a). FTIF stains PEDV antigens in fresh tissues while IHC stains antigen in sections of formalin-fixed tissues. Feces can also be used with an antigen-capturing ELISA to detect virus particle or antigens in a

solid phase by staining with antibodies (Callebaut et al., 1989; De Arriba et al., 2002; Gerber et al., 2014).

Virological tests that detect viral genetic material are referred to as PCR tests. This is the most sensitive diagnostic assay with rapid turnaround for SECoV and can handle a wide range of samples, including rectal swabs, feces, intestinal tissues, intestinal contents, and oral fluids for (Miller et al., 2016; Ren and Li, 2011; Yoon, 2015; Yu et al., 2015; Zhang et al., 2016). A reverse transcription quantitative PCR (RT-PCR), also known as real-time PCR, can enumerate the amount of vRNA in a sample by measuring the cycle threshold (Ct), which has an inverted correlation with the amount of target in the sample. Other PCR assays, including gel-based RT-PCR which amplifies vRNA through use of a specific primer and loop-mediated isothermal amplification (LAMP), which uses multiple primers specific for target DNA performed under isothermal conditions (Ren and Li, 2011). LAMP assays have been used to detect PEDV and differentiate it from other enteric pathogens (Ren and Li, 2011; Yu et al., 2015; Zhang et al., 2016).

Serological assays

Serological assays primarily detect and measure virus-specific antibodies in serum and other bodily fluids, such as colostrum, milk, and oral fluid, and even feces from animals exposed to a pathogen, in this case, SECoV. Upon exposure to SECoV, animals develop a humoral immune response characterized by circulating IgG, IgM, and IgA along with mucosal sIgA. Following re-exposure to homologous SECoV, the mucosal IgA and serum IgG antibody levels are shown to be boosted (Callebaut et al., 1990, 1989; De Arriba et al., 2002; Gerber et al., 2014; Gerber and Opriessnig, 2015; Gimenez-Lirola et al., 2017). Virus-specific serum IgG antibodies are detected starting at 14 – 21 dpi in most SECoV infections, although there can be differences

in antibody kinetics and abundance in neonates (Annamalai et al., 2015; De Arriba et al., 2002; Lanza et al., 1995). For example, sows develop virus-specific antibodies to PEDV and TGEV sooner and sustain significantly higher serum antibody titers longer as antibodies were still detectable 35 – 44 dpi (Gerber et al., 2016). Similarly, other studies observed a higher rate of seroconversion of SECoV-specific IgG in weaned pigs compared to neonates which started at 14 dpi (Gerber et al., 2016; Poonsuk et al., 2016; Saif, 1996; Wesley et al., 1997).

Serological assays commonly used for SECoV include immunofluorescence assay (IFA), serum-virus neutralization test (SVN), and enzyme-linked immunosorbent assay (ELISA). While SVN antibodies are detectable 7-14 days post-infection/challenge, there is no apparent cross-neutralization between PEDV, PDCoV, and TGEV by SVN assay (Lin et al., 2015). However, cross-reactivity does occur between some SECoV on ELISAs. For example, anti-PRCV neutralizing antibodies can confer partial protection against TGEV infection. Such cross-neutralization occurs because PRCV is a deletion mutant of TGEV at the antigenic site of the S protein (Callebaut et al., 1989; Wesley et al., 1997). In addition, S protein is highly immunogenic and can be a target for monoclonal antibodies. In TGEV infection, it has been demonstrated that the blocking of S proteins using monoclonal antibodies results in virus neutralization, thus preventing infection (Kolb et al., 1998; Saif et al., 2012). Similarly, U.S. prototype PEDV and PEDV S-INDEL strains are shown to cross-react in serological most assays and cross-neutralize in-vitro although heterologous protection is only partial in vivo (Chen et al., 2016).

V. Prevention and Control for SECoV

Biosecurity

Implementation of biosecurity practices mitigate the risk of the introduction and spread of a pathogen. Biosecurity is implemented through a set of outlined behaviors by people involved in a specific system which reduce the risk in all activities. In livestock production, biosecurity is crucial in preventing disease outbreaks which can lead to significant economic losses. During the 2013 outbreak, U.S. prototype PEDV spread rapidly across states which caused catastrophic economic losses to the U.S. swine industry. Between 2013-2014, PEDV cost an estimated $900 million - $1.8 billion U.S. dollars in profit loss associated with the greater than 90% pre-weaning mortality and 100% morbidity observed (Paarlberg, 2014). Many studies have been conducted to examine risks that lead to the rapid dissemination of PEDV in the U.S. Of the many possible risks, four main risks were research in detail. First, feed ingredients contaminated with PEDV that were imported from PEDV-endemic countries were suspected to have been the cause of the initial outbreak. While experimentally PEDV-spiked feed was able to survive conditions mimicking shipment to the U.S. from China, pigs did not get sick when fed feed ingredients positive for PEDV vRNA (Dee et al., 2014; Opriessnig et al., 2014). However, once PEDV has broken on a farm, 1 gram of PEDV-infected feces from an infected pig, with a Ct of 37, is enough to contaminate up to 500 tons of feed, in a controlled study (Schumacher et al., 2015). Next, contaminated transport vehicles, such as animal trailers and rendering trucks, were suspected to carry infectious PEDV between farms. A recent study concluded animal trailers that hailed PEDV-infected pigs and were not properly disinfected had the potential carry the residual virus to other farms, suggesting transportation as a possible transmission source between farms (Lowe et al., 2014). The risk of PEDV spread from farm to farm increases as the temporal spatial

distance to the infected farm decreases (Alvarez et al., 2016). Finally, PEDV aerosol transmission was examined when air contaminated with infectious PEDV and vRNA was detected up to 10 miles downwind from the infected farm, suggesting a possible mode of transmission between farms (Alonso et al., 2014). While it is unlikely that processed feed ingredients of pig origin can transmit viable virus, environmental contamination is a high risk for dissemination of infection.

Mitigation

With the PEDV transmission risks discovered, mitigation strategies must be implemented to prevent the spread of disease between farms. An economic evaluation of potential PEDV mitigation strategies were studied. Due to the limited quantity and quality of protective PEDV vaccines, the most cost-effective strategy to control PEDV spread is through a procedure known as "feedback." This practice intentionally controls the exposure of pigs to infectious virus material, generally before parturition to induce protective lactogenic immunity. A recent study assessed the economic advantages to certain biosecurity procedures. The overall losses were decreased by $130,000 U.S. dollars when proper biosecurity procedures were followed. More specifically, replacement gilts were front-loaded, followed by performing feedback and closing off the herd (Weng et al., 2016). This option proved to be cheaper than the cost to vaccinate and more efficacious at preventing or controlling PED. Additionally, mitigation strategies were evaluated to prevent contamination of imported feed ingredients. By proper screening of feed ingredients using diagnostics, the risk of feeding pigs with contaminated feed decreases (Dee et al., 2014; Opriessnig et al., 2014). Overall, better biosecurity practices can help mitigate the risk of emerging SECoV outbreaks.

Vaccination

Vaccination could be a useful prevention strategy, albeit with some obstacles, such as antigenic variation between related SECoV. In the U.S. there are only 2 approved, but not licensed vaccine candidates for PEDV, both killed vaccines (Gerdts and Zakhartchouk, 2016). In China, PEDV is endemic and there are licensed vaccines dating back to 1995 for a killed PEDV-TGEV bivalent vaccine (D. Wang et al., 2016). Over the years, there have been attenuated virus-based vaccines created, including a trivalent vaccine containing TGEV, PEDV-CV777, and porcine rotavirus. While these vaccines do not provide sterilizing immunity, they do help to reduce mortality in suckling piglets infected with SECoV when the piglets uptake colostrum and milk (Gerdts and Zakhartchouk, 2016; D. Wang et al., 2016). However, the antigenic variation between the classical PEDV CV777 and emerging prototype PEDV have made it difficult to protect from mortality losses due to the vaccines inability cross-protect (D. Wang et al., 2016). Vaccine failure to protect against emerging prototype PEDV strains, such as U.S. prototype PEDV, through vaccination with the PEDV CV777 strain has been observed. This is most likely due to a variable region in the S protein in the emerging PEDV strains (X. Wang et al., 2016). In a recently published study, the S gene sequences of known neutralizing epitopes varied between the classical CV777 and prototype strains, which could explain why one vaccine did not cross-neutralize the other (Lin et al., 2015). More research remains to be done to devise efficacious vaccines and vaccination strategies for emerging SECoV.

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CHAPTER 2. COMPARATIVE PATHOGENICITY OF PORCINE EPIDEMIC DIARRHEA VIRUS AND PORCINE DELTACORONAVIRUS IN NURSERY PIGS

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Abstract

In 2013, porcine epidemic diarrhea virus (PEDV) emerged, followed by porcine deltacoronavirus (PDCoV), causing severe diarrhea contributing to a high pre-weaning mortality in naive herds. These newly emerged swine enteric coronaviruses (SECoV), specifically PEDV and to lesser extent PDCoV, caused $481-$929 million U.S. dollars to U.S. swine industry. While SECoV is well characterized in young piglet models, there has been no study to compare pathogenicity of these two viruses. In the present study, pathogenicity of PEDV and PDCoV was evaluated simultaneously in nursery pigs. Six-week-old pigs naïve for SECoV, including transmissible gastroenteritis, were allotted to the following treatment groups: 1) sham-inoculated control; 2) PEDV-inoculated; 3) PDCoV-inoculated. Clinical signs were monitored daily. Animals were serially necropsied with tissues, serum, and intestinal content collected post-mortem. All tissues were examined for histopathology and viral antigen. Additionally, serum and fecal swabs were periodically collected until the study was terminated at 42 days post inoculation (dpi). Quantitative RT-PCR (qPCR) was used to measure viral RNA (vRNA) in feces, serum, and tissues. Diarrhea did not persist past 5 dpi for PEDV-inoculated and 7 dpi for PDCoV-inoculated groups, but PEDV vRNA was shed longer and at higher mean viral titers than

PDCoV. Similarly, PEDV vRNA was detected at higher levels in small and large intestinal tissues and gut-associated tissues when compared to PDCoV. Microscopic lesions were detected in the small intestinal tissues of PEDV-inoculated, but not PDCoV-inoculated groups, despite detection of both PEDV and PDCoV vRNA in intestinal tissues. Lesion scores in the small intestine peaked for PEDV-infected pigs at 5 dpi. Indirect fluorescent antibody and serum-virus neutralization tests detected serum antibodies starting at 7 dpi, with the majority of seroconversion by 14 dpi PEDV-inoculated and PDCoV-inoculated groups. Antibody detection continued until the end of the study for PEDV-inoculated, but not PDCoV-inoculated animals. In summary, PEDV appears to be more virulent than PDCoV in growing pigs under the conditions presented in this study.

Introduction

Porcine epidemic diarrhea virus (PEDV) emerged in U.S swine population for the first time in 2013 causing a devastating epidemic. (Stevenson et al., 2013). PEDV belongs to the family *Coronaviridae*, genus *Alphacoronavirus*, along with other swine coronaviruses, such as transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (Ma et al., 2015; Su et al., 2016). PEDV which caused a devastating diarrheal outbreak across several European countries was first isolated in 1978 (Pensaert and De Bouck, 1978). Originally, PEDV infection was mistaken for a TGE-like outbreak due to their similarities in clinical presentation. However, the outbreak was later determined to be caused by a new coronavirus, PEDV, due to its lack of serological similarity to TGEV (Pensaert, 1992; Pensaert and De Bouck, 1978). During the 2013 U.S. outbreak, PEDV caused severe diarrheic disease in pigs of all ages with 100% morbidity. Mortality was mostly associated with suckling piglets and approached greater than 90% in naïve herds (Jung et al., 2016a; Madson et al., 2014; Stevenson et al., 2013). Today, PEDV remains

endemic in the U.S. with a lower incidence and mortality rate associated with previously-exposed herds, suggesting presence of herd immunity.

Shortly after the emergence of PEDV, detection of another coronavirus, now named porcine deltacoronavirus (PDCoV), was first reported in U.S. swine herds in 2014, although the virus may have been introduced to the U.S. in 2013 according to a diagnostic laboratory based retrospective study (Sinha et. al., 2015). PDCoV belongs to the genus, *Deltacoronavirus*, that comprises many deltacoronaviruses identified in both avian and mammalian species (Woo et al., 2012). During the PDCoV outbreak in U.S. swine herds, PDCoV viral RNA was detected in clinical specimen from both sows and piglets (Wang et al., 2014). PDCoV is believed to attribute to the economic consequence associated with the 2013-2014 PEDV outbreak, with up to 40% mortality associated with PDCoV infection of naïve pre-weaning piglets (Jung et al., 2016a). Today, PDCoV, similar to PEDV, is also endemic in the U.S.

PEDV and PDCoV cause gastrointestinal infection in pigs leading to diarrhea, similar to TGEV, denoting all of them as swine enteric coronaviruses (SECoV) due to the similarity in clinical presentation caused by their infections. SECoV are mainly transmitted through fecal-to-oral route from infectious pigs (Chen et al., 2014; Saif and Wesley, 1992). There are differences that exist in the pathogenicity of PEDV and PDCoV, which lead to diminished severity of infection in PDCoV-infected piglets versus a more severe disease caused by PEDV (Chen et al., 2015; Jung et al., 2016; Lin et al., 2016). PEDV viral shedding peaked during the first week of clinical disease, with viral shedding diminished after 14 dpi (Crawford et al., 2015). In 5-day-old piglets, PEDV viral RNA can be detected in the feces of infected pigs up to 14 dpi versus 26 dpi in 21-day-old pigs (Madson et al., 2014; Niederwerder et al., 2016; Thomas et al., 2015). In

comparison, PDCoV can be shed in the feces of 10-day-old infected piglets for up to 10 dpi (Jung et al., 2016a).

SECoV can infect pigs of all ages, but mortality and severe disease is most commonly associated with neonate or suckling pigs (Lin et al., 2015; D.M. Madson et al., 2014; Saif, 1999; Joseph T. Thomas et al., 2015). Severe clinical disease caused by PEDV and PDCoV is characterized by the presence of pathological lesions in the small intestine. Pathological changes occur in the affected tissues causing both shortening of small intestinal villi and erosion of epithelial cells on the tips of the villi. Grossly, the small intestine of the affected pigs was thin-walled and distended with the presence of yellow to grey liquid contents. Viral antigen was detected in both the villous tips and crypts of the small intestine, although the spread to the villous crypts is not common with PDCoV infection (Jung, Saif, 2016; Madson et al., 2014b; Stevenson et al., 2013). On-set of clinical disease manifested by diarrhea, lethargy, and vomiting occurred as early as 12-18 hours post infection for PEDV (Madson et al., 2014; Saif et al., 2012). On-set of diarrhea in PDCoV infected pigs is similar to PEDV, however, vomiting is not usually seen (Chen et al., 2015; Jung et al., 2016b; Ma et al., 2015).

Overall, SECoV pathogenicity is affected by both age and type of infection, however, most of the comparative pathogenicity studies have focused on only PEDV or PDCoV infections in neonate animals. There has not been a pathogenicity study done to compare PEDV and PDCoV infections in older animals. The purpose of this study was to evaluate the comparative pathogenicity of PEDV and PDCoV in nursery pigs.

Materials and Methods

Animals, Housing, and Experimental Design

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Iowa State University (IACUC# 11-14-7903-S). Seventy-five SECoV naïve 3-week-old weaned pigs (Choice Genetics) were purchased from a commercial herd and brought to Iowa State University's animal facility. When piglets reached 6 weeks of age they were allotted to 3 treatment groups: 1) Sham-control, 2) PEDV inoculated, and 3) PDCoV inoculated. All animals were screened prior to and after arrival and determined negative for TGEV, PEDV, and PDCoV by serology and qPCR. Animal weights were recorded prior to and during the experiment. Across each treatment, either 8 or 17 animals were assigned to 8 pens over a 42-day testing period. Using a randomized block design, 4 pigs/group were allotted at each time point for necropsy and individual animals were samples across all groups. At 0 dpi, all animals were inoculated with 5 ml of Sham, PEDV, or PDCoV via gastric gavage. All animals were housed at the Livestock Infectious Disease Isolation Facility operating at BSL2 compliance, with each treatment group separated into designated rooms and pens with individual ventilation and washing systems.

Virus Inoculum

Porcine epidemic diarrhea virus isolate US/Iowa/18984/2013 (Hoang et al., 2013) and porcine deltacoronavirus strain US/Iowa/25573/2014 were used for the study. Both isolates were made from clinical cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL, Ames, IA), plaque-cloned before further propagation. The PEDV isolate was propagated in Vero cells (ATCC® CCL-81, Manassas, VA), while the PDCoV isolate was propagated in swine testicular (ST) cells (ATCC® CRL-1746, Manassas, VA). The titer of each

virus represented by the median tissue culture infectious dose ($TCID_{50}$/ml) was determined using a microtitration infectivity assay in the respective cell lines for each virus, then adjusted to 1 x 10^3 $TCID_{50}$/ml for inoculation. Virus-free cell culture supernatant as Sham inoculum was prepared in the identical manner alongside with virus inoculum.

Clinical Assessment

After inoculation, animals were monitored daily for signs of clinical disease. Body weight and feed intake were recorded prior to inoculation and throughout the course of the experiment. Average daily gain (ADG), average daily feed intake (ADFI), and growth to feed ratios (G:F) were calculated using weekly pen feed intake and body weight changes. Daily fecal samples were taken daily for 1 week, then weekly thereafter and any changes in the fecal consistency were recorded.

At necropsy, intestinal tissues and non-intestinal tissues were assessed for gross lesions and placed neutral buffered formalin for histopathology and immunohistochemistry (IHC). Intestinal contents were observed for color and texture changes consistent with malabsorptive diarrhea.

Sample Collection, Processing, and Storage

Four or eight pigs from each treatment group were selected and necropsied at 2, 5, 7, 14, and 42 dpi. Colon, cecum, duodenum, jejunum, ileum, mesenteric lymph node, stomach, esophagus, spleen, kidney, lung, and tracheobronchial lymph node tissue samples and whole blood were collected at each necropsy. Feces, serum, and pen-based oral fluids were taken daily from 0 to10 dpi, and weekly thereafter until the end of the study (42 dpi). Tissue homogenates and fecal processing were performed on fresh necropsy samples as described previously (Curry et al., 2017; Sinha et al., 2015). Sections from each fresh tissue were formalin-fixed, then

embedded into paraffin blocks as previously described (Madson et al., 2014). Whole blood that was allowed to clot overnight was centrifuged and serum was collected, then frozen until use in serological assays. Oral fluid samples were collected as previously described (Poonsuk et al., 2016), then stored frozen until sample evaluation could be performed. Once processed, all samples were aliquoted into 2 ml aliquots and placed in -80° C until testing was performed.

Viral RNA Extraction

Tissue homogenates, processed feces, and serum were used for in quantitative reverse transcription polymerase chain reaction (RT-qPCR) testing. For viral RNA (vRNA) extraction, the MagMax™ viral isolation kit and a Kingfisher™ 96 instrument (ThermoFisher Scientific, Waltham, MA) were used with a modification for high volume RNA extraction approved and utilized in the ISU-VDL (SOP# 9.3833v2). RNA extracts were used immediately for qPCR assay and any remaining extract was stored at -80° C. Virus standards with known viral titers ($TCID_{50}$/ml) were prepared using serial dilutions of virus stock from challenge inoculum and extracted with samples as described above.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction Assays (qPCR)

Virus-specific qPCR was performed on extracts from tissues, feces, and serum for detection of virus genetic material. Positive and negative extracts were used during each run for validation of assays. PEDV and PDCoV qPCRs were performed as previously described (Sinha et al., 2015; Thomas et al., 2015). Briefly, PEDV nucleocapsid (N) gene primers and probe were designed from PEDV sequences assessed through GenBank (accession no. KF272920) and PDCoV membrane (M) gene primers and probe were adopted from published work (Sinha et al., 2015; Woo et al., 2012) were used in combination with Path-ID™ Multiplex One-Step RT-PCR kit (ThermoFisher Scientific, Waltham, MA) on all extracts by following manufacturer's

recommended procedure. Cycle threshold (Ct) cut-offs for positive samples were < 37 and > 40, respectively, with suspect Ct values ranging from 37 – 40.

Histopathology and Immunohistochemistry

Tissues were collected fresh and placed in 10% neutral buffered formalin for up to 48 hours before being trimmed, processed, and embedded into paraffin, as mentioned previously (Madson et al., 2014). Four micron thick sections were cut and stained in duplicates with hematoxylin and eosin as per routine procedure at the ISU-VDL. Non-intestinal tissues were evaluated for evidence of systemic inflammation by a veterinary pathologist blinded to the treatment group and dpi at the ISU-VDL. Small intestinal sections were identified by location, and villous length and crypt-depth were evaluated blindly by a veterinary pathologist at the ISU-VDL (Madson et al., 2014).

Paraffin-embedded tissue sections were used for immunohistochemistry (IHC) analysis. Briefly, for PEDV IHC, formalin-fixed, paraffin-embedded (FFPE) tissue sections were mounted and processed as described previously (Madson et al., 2014) and stained using a commercially available PEDV N protein-specific monoclonal antibody 6C8 (BioNote, Seoul, Korea). Similarly, PDCoV IHC included FFPE sections that were processed and stained using a M protein-specific monoclonal antibody as mentioned previously (Okda et al., 2016). IHC scoring, as described previously (Madson et al., 2014; Thomas et al., 2015), was used with the following criteria: 0 = no staining; 1 = approximately 1-10% enterocytes with antigen positive staining; 2 = approximately 10-25% positive staining; 3 = approximately 25-50% positive staining; 4 = approximately 50-100% positive staining.

Serological Assays

Virus-specific seroconversion was measured using indirect fluorescent antibody (IFA) and serum-virus neutralization (SVN) assays. For IFA assay, 96-well plates with a confluent Vero (PEDV) or ST (PDCoV) monolayers were used. The respective cells were inoculated with 100 µl/well of PEDV (US/Iowa/18984/2013) or PDCoV (US/Iowa/25573/2014) viruses at 10^3 $TCID_{50}$/ml and incubated at 37° C for 1 hour in a 5% CO_2 incubator. Following a 1-hour incubation, the inoculum was replaced by maintenance media, DMEM GlutaMAX® (ThermoFisher Scientific, Waltham, MA), with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 5 µg/ml trypsin, 1X Antibiotic-Antimycotic (Sigma-Aldrich, St. Louis, MO). Plates were placed back in the incubator for 24 hours. Once >50% cytopathic effect (CPE) was observed, media was removed from plates and cells were fixed with 80% cold acetone aqueous solution for 10 minutes at ambient temperature. Acetone was discarded and plates were air dried, then stored at -20 degrees C until used. On day of testing, serum was serially diluted starting at 1:4 dilution followed by 2-fold serial dilutions in 1X phosphate-buffered saline (PBS) at pH 7.4 (ThermoFisher Scientific Waltham, MA) and 50 µl of each diluted sample was added in duplicate to each well on the plates, once they reached room temperature. Plates were incubated for 1 hour in a 37° C chamber incubator. After incubation, plates were washed 4 times using PBS containing 20% Tween, then tapped dry. Optimally diluted Fluorescein isothiocyanate (FITC) conjugated goat anti-swine IgG antibody (Bethyl Laboratories Inc., Montgomery, TX) was added and plates were incubated for 30 minutes in the dark at 37° C. Plates were washed, tapped dry, and then read under 20X magnification using a fluorescence microscope (Olympus Scientific Solutions Americas Corp., Waltham, MA). Each well was determined to be positive or negative

based on the similarity to the staining and fluorescence patterns of the positive and negative control wells. The cutoff titer for positive samples is 1:8.

For SVN assay, 96-well plates were prepared with confluent Vero (PEDV) or ST (PDCoV) cell monolayers. Serum samples were heat-inactivated in a 56^{o} C water bath for 30 minutes and were serially diluted in PBS starting at 1:2 ratio. One hundred µl of each diluted serum were added in duplicate to each well containing confluent cell monolayers. Virus inoculum (100 µl/well) was also added to the plate wells with samples and incubated at 37^{o} C for 1 hour in a 5% CO2 incubator. Following a 1-hour incubation, the inoculum was replaced by the maintenance media. Plates were placed back in the CO_2 incubator and observed for signs of CPE. Plates were read between 24-120 hours later. SVN antibody titer was determined by the inverse of the highest serum dilution that inhibited virus replication (i.e. CPE absent). SVN cutoff titer for positive samples was 1:8. After recording results, cells were fixed and stored at -80^{o} C for later confirmation by IFA.

Statistical Analysis

Data was analyzed using analysis of variance (ANOVA) with a p-value of ≤ 0.05 considered to be significant (GraphPad PRISM 7®, GraphPad Software, La Jolla, CA). Tukey multiple comparisons were used to determine statistical difference between treatment groups if responses were considered significant. Viral titers ($TCID_{50}$ equivalent) were converted to $\log_{10}$ values for analysis.

Results

Clinical Assessment and Fecal Shedding

Diarrhea occurred as early as 1 dpi for pigs receiving either PEDV or PDCoV inoculum with resolution occurring after 6 dpi. As measured by PCR, PDCoV was shed in the feces of infected pigs until 5 dpi, while PEDV was shed in the feces until 7 dpi, despite resolution of diarrhea by 6 dpi. Based on qPCR estimation, the mean PEDV titer in feces peaked at 5 dpi and was significantly higher than PDCoV titers in feces during the first week of infection (Figure 2.1). In comparison, PDCoV titers in feces peaked at 3 dpi, sooner than PEDV titers (Figure 2.1).

Grossly, both PEDV- and PDCoV-inoculated pigs displayed thin-walled distended small intestines containing liquid contents at 2 dpi. Colon contents were also liquid and effusive. At 5 dpi, animals from both virus-infected groups continued to exhibit signs of distended thin-walled small intestines and watery fecal contents. Fecal contents returned to a normal consistency starting at 5 dpi for PDCoV-inoculated and 7 dpi for PEDV-inoculated pigs. No diarrhea was observed in any of sham-inoculated negative control animals at any point in the experiment. Additionally, all control tissues had normal appearance and fecal contents remained normal during the entire study.

Body Weight and Feed Intake

While body weight did not decrease as a result of PEDV or PDCoV infection, PEDV-inoculated pigs suffered from a significant ($p < 0.05$) reduction in average daily gain during the first week of the clinical disease, affecting the overall total body weight gained at 42 dpi significantly ($p = 0.012$). Average daily feed intake (ADFI) was also reduced in the PEDV-inoculated group until 7 dpi. In contrast, PDCoV-inoculated animals did not gain weight differently from the control pigs. Similarly, no differences were seen in the ADFI between the

PDCoV-inoculated or negative control groups. Detailed growth performance data from PEDV- or PDCoV-infected pigs has been published by a collaborating research group (Curry et al., 2017).

Histopathology

Microscopic lesions consistent with viral enteritis were not observed in intestinal tissues from the negative control group. On the other hand, PEDV-inoculated animals exhibited enteritis lesions within the small intestinal tissues, with the average lesion score of 1 or greater from 2 to 5 dpi (Figure 2.2). At 5 dpi, all of the small intestinal tissue sections from PEDV-inoculated pigs had lesions with a mean lesion score of one or greater. The highest mean lesion score occurred in the PEDV group at 5 dpi with an average lesion score of 2.6, whereas no lesions were observed after 7 dpi and remained negative through the remainder of the study (Figure 2.2). In contrast, no lesions were found in small intestinal tissue from PDCoV-inoculated pigs or large intestinal tissue from both virus groups (Figure 2.2). There were no signs of microscopic lesions or inflammation in non-intestinal tissues.

Viral Load in Intestinal Tissues

All the small intestinal segments were positive for PEDV or PDCoV vRNA by qPCR based on the groups corresponding inoculum (Figure 2.3). In the duodenum and jejunum, the mean PEDV titer was greater at 2 and 5 dpi than the corresponding PDCoV titer (Figure 2.3 A-B). However, PDCoV vRNA titers continued to be detected at a low level until 14 dpi in the duodenum (Figure 2.3A). Similarly, the mean PEDV titer in the ileum was significantly ($p < 0.05$) greater than the PDCoV titer at 5 dpi (Figure 2.3C). This trend was similar among PEDV-infected small intestinal tissues throughout the first week post inoculation.

vRNA was also detected in some portion of the large intestine. PEDV vRNA was detected in the colon starting at 2 dpi, with the mean viral titer quickly declining and becoming negative after 7 dpi. At 5 dpi, the mean PEDV titer was higher than PDCoV titer in the colon, although it was not statistically significant (Figure 2.3D). In contrast, cecum tissues were negative by qPCR for both viruses throughout the study period.

Viral RNA Detection in Non-Intestinal Tissues

Both PEDV and PDCoV vRNA were detected outside of the intestinal tissues which included the mesenteric lymph node (MLN) and stomach. All other non-gut tissues (esophagus, spleen, kidney, lung, and tracheobronchial lymph node) tested negative for viral RNA detection by qPCR.

The mean viral titer was similar between PEDV and PDCoV groups, but peak titers occurred at different times (Figure 2.4). In the MLN, the mean PEDV titer peaked at 5 dpi which was similar to the observations from intestinal tissues. In comparison, the mean PDCoV titers in the MLN peaked at 2 dpi (Figure 2.4A). In the stomach, both PEDV and PDCoV titers peaked at 7 dpi which was later than seen in the intestinal tissues (Figure 2.4B). Interestingly, PDCoV persisted until 14 dpi which was longer than PEDV in the stomach (Figure 2.4B).

Viremia

PDCoV-inoculated pigs were not viremic, whereas PEDV-inoculated pigs had detectable viral titers in the serum at 2 to 7 dpi (data not shown).

Serum Antibody Response

While negative control animals remained seronegative by both IFA and SVN assays during the duration of the study, PEDV- and PDCoV-inoculated pigs began seroconversion to their corresponding viruses by 5 dpi. At 7 dpi by IFA and 14 dpi by SVN, more than 50% of the

animals seroconverted (Table 2.3). All of the PEDV-inoculated pigs developed neutralizing antibodies by 28 dpi and remained seropositive until 42 dpi as measured by SVN, while PDCoV antibodies were developed in 50% of the PDCoV-inoculated pigs at 14 dpi (Table 2.3). In contrast to PEDV antibody response, PDCoV serum antibodies were detected at much lower titers by both IFA and SVN with antibodies waning and no longer detectable at 21 dpi. (Figure 2.5**)**.

Discussion

Pathogenicity and pathogenesis of either PEDV or PDCoV have been well characterized in pre-weaning pigs. However, as these viruses can infect pigs of all ages, our study objective was to compare the pathogenicity of PEDV and PDCoV in older post-weaned (6 weeks of age) pigs under the same conditions. Published as part of a collaborating study (Curry et al., 2017), PEDV-inoculated pigs experienced a 23% reduction in average daily gain during the first week after inoculation when compared with PDCoV-inoculated and control groups. In the present study, severe histological lesions were found in the small intestinal tissues of PEDV-, but not PDCoV-inoculated pigs. Additionally, the viral load was greater in tissues and feces of PEDV-infected pigs than seen with PDCoV infection. Overall, our data suggests that PEDV is more virulent than PDCoV in growing pigs under the conditions presented in the study.

PEDV infection in growing animals has shown to cause changes in the small intestinal morphology of growing pigs (Curry et al., 2016). More specifically, reduction of Claudin 2 expression in the jejunum was observed in PEDV tissues sections suggesting the gut barrier has become compromised or “leaky.” Morphological changes can occur as a result of PEDV infection of enterocytes. In the jejunum, PEDV-infected enterocytes displayed shortening of villus height, with the most severe changes occurring between 2 to 5 dpi as reported by our

collaborating study (Curry et al., 2016). In PDCoV-infected suckling piglets, vacuolation of infected enterocytes were shown by immunofluorescence assay; however, no evidence of cell death caused by apoptosis or cell lysis occurred as a result of the infection (Jung et al., 2016b). Collectively, PEDV appears to induce more severe lesion in the small intestinal wall than PDCoV. Furthermore, previous studies show more severe pathogenicity in pre-weaning piglets when compared to the pathogenicity in growing pigs as shown in the present study (Chen et al., 2016; Lin et al., 2016; Thomas et al., 2015).

While the present study showed that both PEDV and PDCoV were pathogenic to growing pigs, the extent of virus replication was different between the 2 viruses in growing pigs. PEDV load in the tissues and feces was significantly greater than that of PDCoV. Additionally, PEDV vRNA was detected at higher levels in the colonic tissues compared to PDCoV, which was predominantly found at moderate levels in the small intestinal tissues, indicating that for some reason the PEDV strain used replicated in growing pigs better than the PDCoV strain used in this study. Such difference may have influenced lesion development in the intestine as PEDV, but not PDCoV, caused lesions in the intestinal tissues, supporting the difference in the extent of infection between the two viruses. PEDV has been shown to replicate in both the villous and crypts enterocytes while PDCoV is most commonly found in the villous enterocytes, which could lead to the difference in the extent of viral replication in the intestinal tissues (Curry et al., 2016; Jung, Saif, 2016; Saif et al., 2012). In a study conducted to evaluate the minimum infectious dose (MID) of PEDV, the PEDV MID for 21-day-old weaned pigs was at least 10-fold higher amount of virus than the MID required to infect 5-day-old pigs (Thomas et al., 2015), suggesting that more PEDV is required to cause severe disease in older pigs. Unfortunately, there have been no studies done to evaluate the MID of PEDV or PDCoV for growing pigs, which

would need to be performed to understand the difference in the pathogenicity of PEDV and PDCoV in growing pigs observed in this study.

Serological assays that measure antibody specific for PEDV and, to a lesser extent, PDCoV in swine serum, milk, colostrum, and oral fluids have been developed (Chen et al., 2016b; Lin et al., 2015b; Poonsuk et al., 2016). Of these assays, IFA assay and ELISA have been commonly used for the detection of virus-specific IgG and IgA, while SVN assay detects neutralizing antibodies which may or may not be IgG. This study used IFA and SVN assays to access duration of seroconversion and antibody levels in serum because a PDCoV ELISA was not available for the present study. No cross-reactivity was observed between PEDV and PDCoV in the IFA and SVN assays. IFA antibodies tended to develop earlier and disappear faster than SVN antibodies against the viruses, probably reflecting the difference in antigen representation and target antigens between the 2 assays. Interestingly, the antibody response of pigs against PEDV and PDCoV differed in our study. PEDV-specific antibodies were detected at higher titers and persisted significantly longer than PDCoV-specific antibodies. Strikingly, PDCoV-specific neutralizing antibodies waned after only 21 dpi, while PEDV-specific neutralizing antibodies continued to be detected in all PEDV-inoculated animals until the end of the study. The early disappearance of PDCoV-specific antibodies could be explained by a lack of good and continuous antigenic stimulation affecting systemic antibody response to the virus, which may coincide with relatively poor replication of the virus in the pigs as shown in the study. Poor antigenic stimulation would not activate the appropriate co-stimulatory molecules required for a robust antibody response. As a result, there would be a lack of antibodies in response to the PDCoV infection (Murphy et al., 2012; Saif, 1996). Alternatively, virus-infected cell killing mediated by cytotoxic T lymphocytes, which release co-stimulatory molecules to activate other

lymphocytes, including B cells. An absence of robust antigenic stimulation could explain a weakened T cell response, hence, the absence of antibodies later in infection. While no studies have been done to assess the cell-mediated immune response against PDCoV, another swine pathogen, swine influenza A virus, has been studied extensively. A recent study shows a robust T cell response mediates protection from subsequent infections and can facilitate better immune protection from unrelated strains of influenza (Olson et al., 2017; Sridhar et al., 2015). It is possible that PDCoV infection could not induce a strong T cell response in older weaned pigs, however, studies need to be conducted to test this hypothesis. In summary, more information is needed to determine the mechanism causing differences in PEDV and PDCoV antibody responses in growing pigs.

In summary, both PEDV and PDCoV were pathogenic in growing pigs under the conditions of this study. However, the extent of the viral load in tissues and feces and severity of disease and lesions was more severe in PEDV-infected pigs when compared to PDCoV-infected pigs even though virus dissemination was similar among the two viruses. Furthermore, PEDV-infected, but not PDCoV-infected, pigs developed a robust serum antibody response which developed 1-to-2 weeks post inoculation, suggesting better antigenic stimulation from PEDV most likely due to better replication than PDCoV under conditions presented in the study. Overall, PEDV can cause more virulent disease than PDCoV in growing pigs and have greater clinical impact which should be taken into consideration for prevention and control of SECoV.

Acknowledgements

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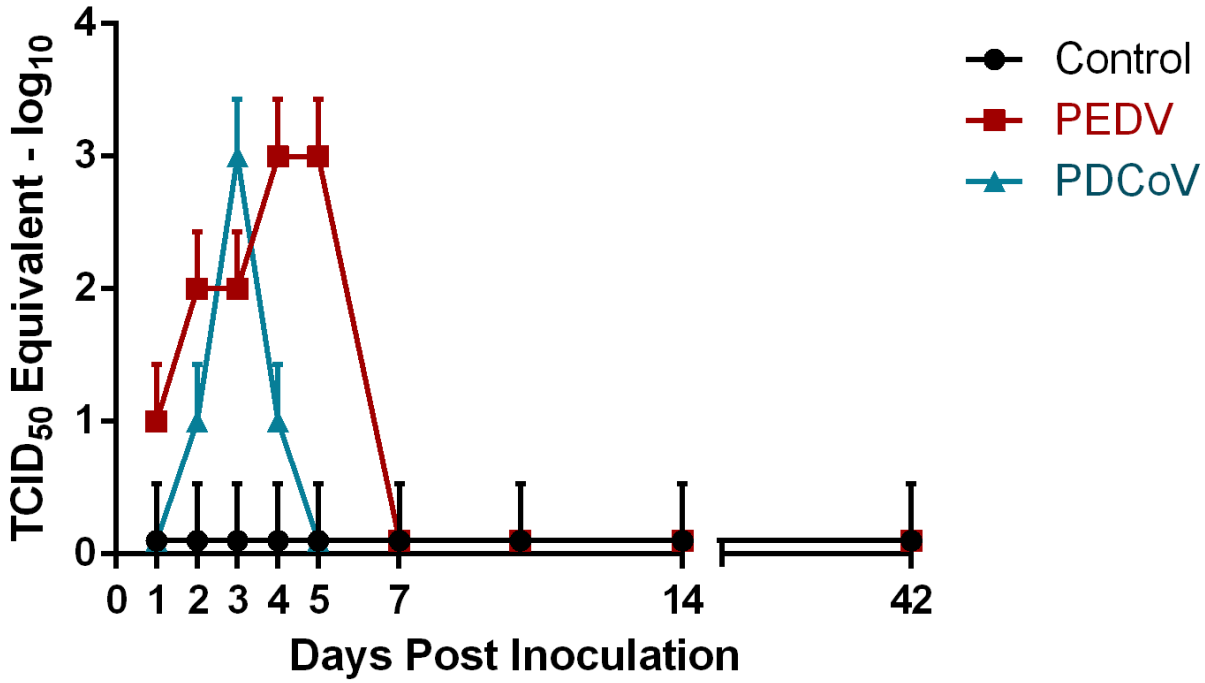

Figure 2.1 ***Fecal shedding of porcine epidemic diarrhea virus (PEDV, red rectangle) and porcine deltacoronavirus (PDCoV, blue triangle) from pigs orogastrically inoculated with each virus at the rate of 10^3 $TCID_{50}$/ml compared with sham-inoculated control pigs (black circle) over time as estimated by quantitative RT-PCR (p-value < 0.001).***

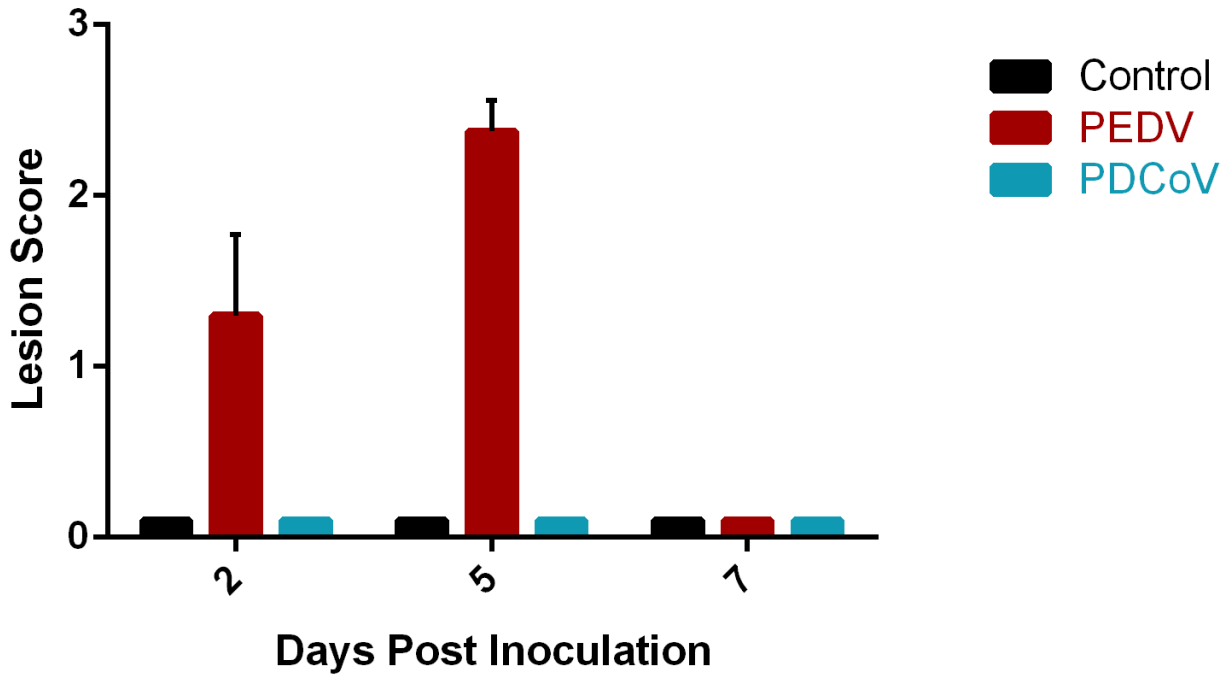

Figure 2.2 ***Scores of microscopic lesions in small intestine of porcine epidemic diarrhea virus (PEDV)-inoculated (red bars) and porcine deltacoronavirus (PDCoV)-inoculated (blue bars) pigs necropsied at 2, 5, and 7 days post inoculation as compared to Sham-inoculated control pigs (black bars). Lesion scores of 0 are considered negative, while lesion scores greater than 1 are considered positive with increasing degrees of lesions in affected tissues at the following rates: (1 = 1-25%; 2 = 26-50%; 3 = 51-75%; 4 >75%). Each bar represents the mean lesion score of 4 pigs per group across different necropsy time points.***

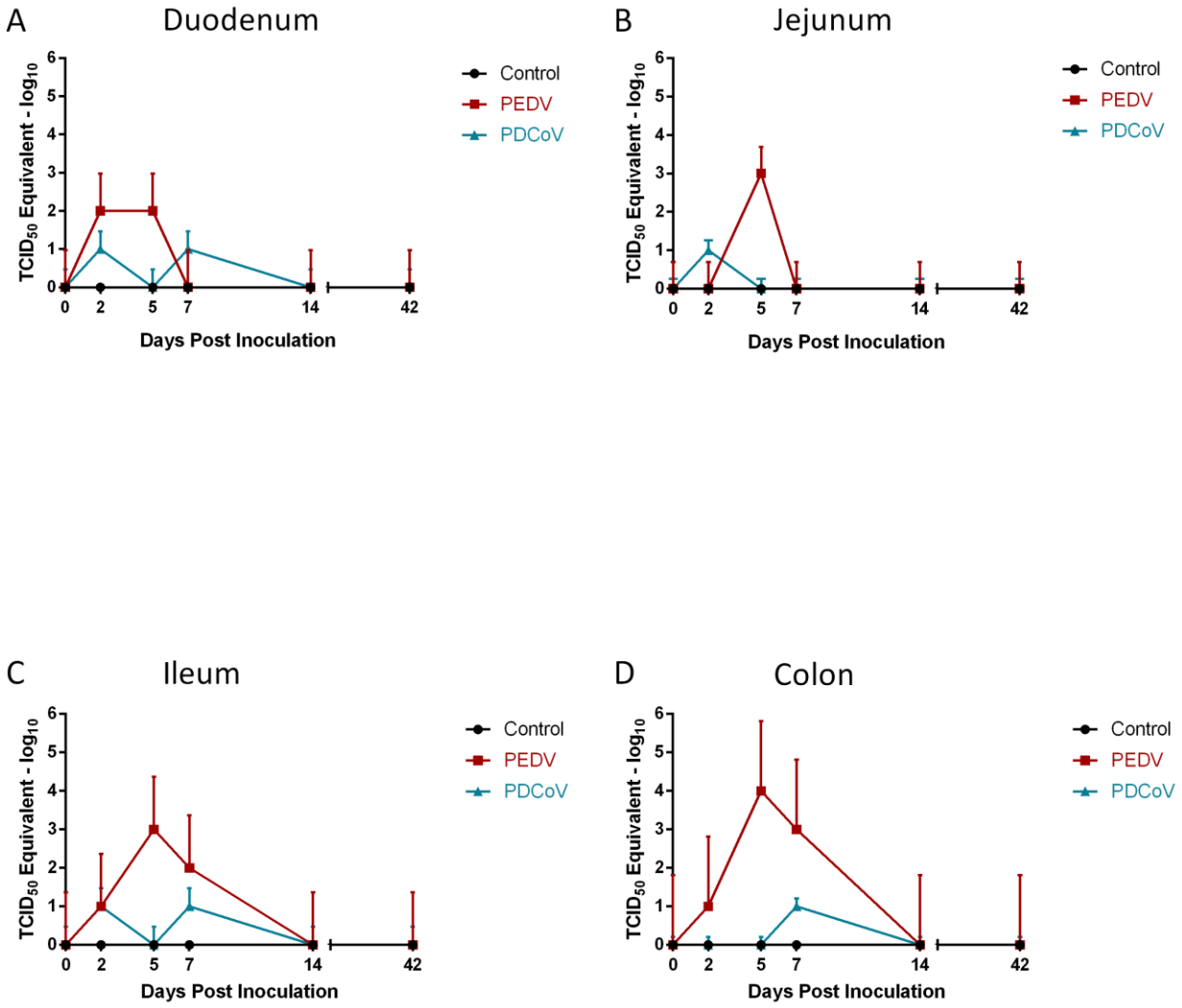

Figure 2.3 ***Mean porcine epidemic diarrhea (PEDV, red triangle) or porcine deltacoronavirus (PDCoV, blue triangle) RNA titers in intestinal tissues of pigs orogastrically inoculated with each virus at the rate of 10^3 $TCID_{50}$/ml compared with sham-inoculated control pigs (black circle) over time as estimated by quantitative RT-PCR (p-value < 0.001).***

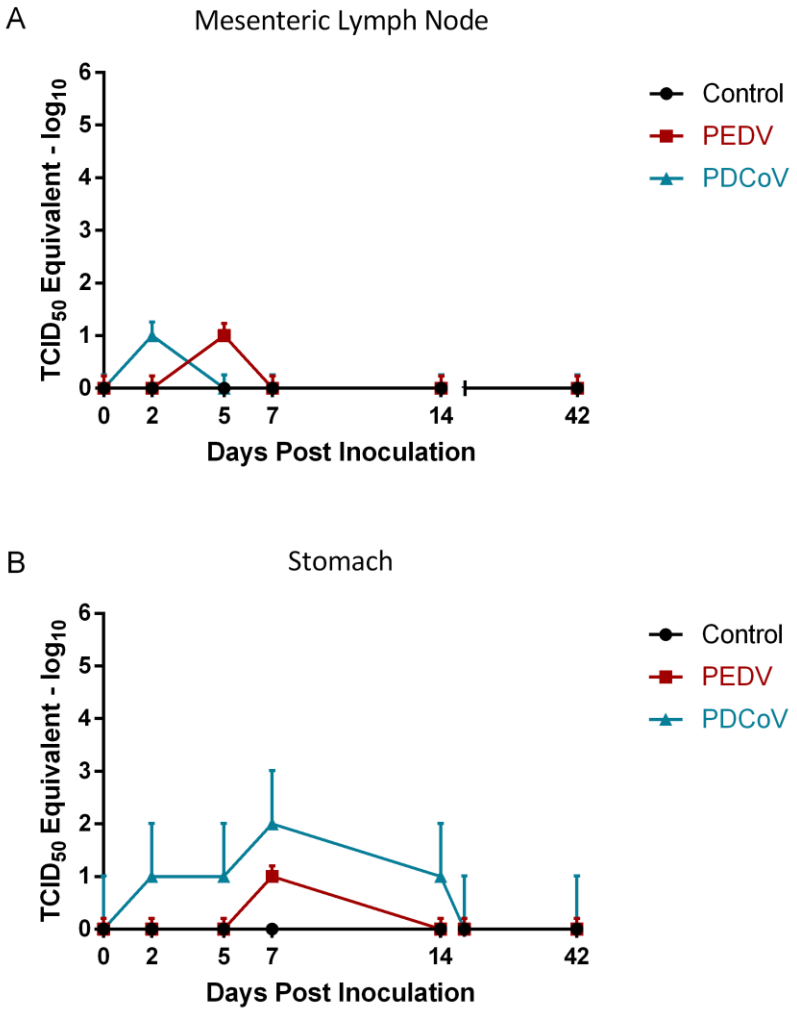

Figure 2.4 ***Mean porcine epidemic diarrhea virus (PEDV, red rectangle) and porcine deltacoronavirus (PDCoV, blue triangle) RNA titers in non-intestinal tissues from pigs orogastrically inoculated with each virus at the rate of 10^3 $TCID_{50}$/ml compared with sham-inoculated control pigs (black circle) over time as estimated by quantitative RT-PCR (p-value < 0.001).***

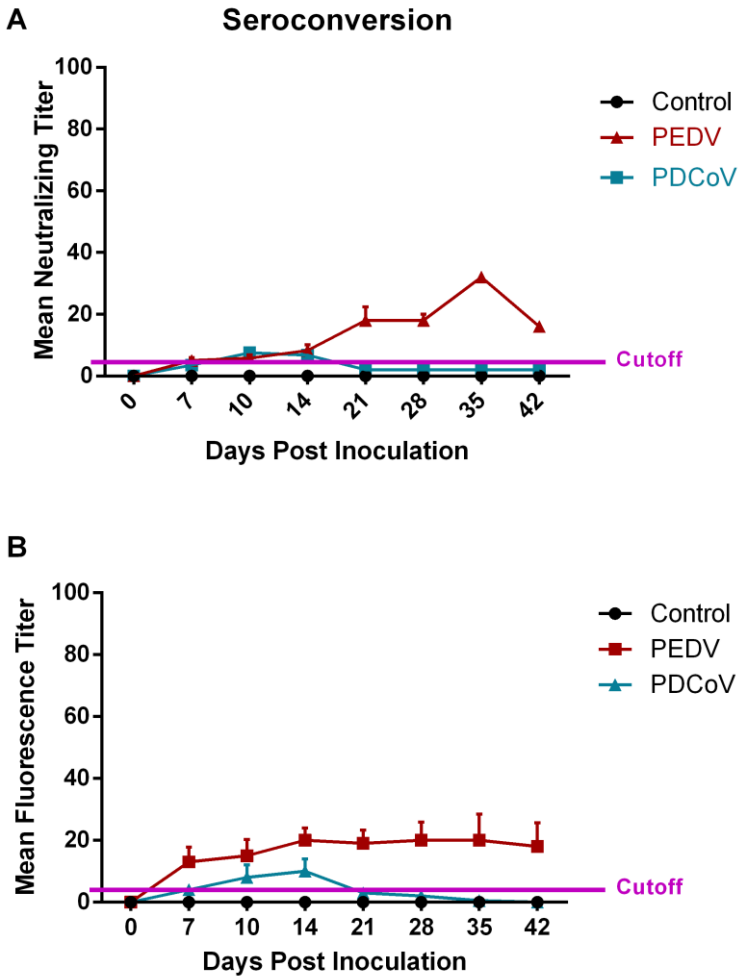

Figure 2.5 ***Serum fluorescence antibody (A) and virus neutralizing antibody (B) from the serum of pigs inoculated orogastrically with porcine epidemic diarrhea virus (PEDV) (red, $p < 0.001$) or porcine deltacoronavirus (PDCoV) (blue, $p > 0.05$) at 10^3 $TCID_{50}$/ml over time compared with sham-inoculated control pigs (black).***

Table 2.1 Virus shedding in feces collected from pigs inoculated with PEDV or PDCoV

	dpi[2]						
Item	1	2	3	4	5	7	SD
Cycle threshold[1]							
Control	40.00	40.00	40.00	40.00	40.00	40.00	0.00
PEDV	34.73	31.98	30.12	27.49	27.86	40.00	4.33
PDCoV	40.00	39.07	32.98	38.43	39.91	40.00	2.49

[1]Average cycle threshold of all pigs Ct ≥ 40 are considered PCR negative

[2]Days post inoculation

Mean Ct values of PEDV and PDCoV from pigs orogastrically inoculated with each virus at the rate of 10^3 $TCID_{50}$/ml compared with Sham-inoculated pigs (control) over time as estimated by quantitative RT-PCR.

Table 2.2 Percent Positive Serum Antibody in Pigs

	dpi[2]								
Item	0	2	5	7	14	21	28	35	42
IFA Percent Positive									
Control	0	0	0	0	0	0	0	0	0
PEDV	0	0	56	60	67	67	50	50	50
PDCoV	0	0	59	25	20	17	13	0	0
Serum Virus Neutralization Titer[1]									
Control	0	0	0	0	0	0	0	0	0
PEDV	0	0	14	32	63	83	100	100	100
PDCoV	0	0	16	38	50	42	0	0	0

[1]Average serum neutralizing antibody titer among all pigs

[2]Days post inoculation

Proportion of seropositive pigs for porcine epidemic diarrhea virus (PEDV) or porcine deltacoronavirus (PDCoV) as measured by indirect fluorescent antibody (IFA) assay and serum-virus neutralization (SVN) assay at each sampling time (dpi) after experimental infection with each virus via orogastic route.

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CHAPTER 3. GENERAL CONCLUSIONS

General Discussion

Historically, TGEV, an alphacoronavirus discovered in 1946, was a major cause of severe diarrheic disease in young pigs, but is less common today in the U.S. In 2013, PEDV, a novel coronavirus, caused a significant outbreak of viral diarrhea U.S. swine herds. In 2014, PDCoV, another new deltacoronavirus, was detected as a causal agent of diarrheic disease in sows and piglets for the first time in the U.S. The recent emergence of new SECoV caused $481 to $929 million U.S. dollars, a devastating economic impact on the U.S. swine industry (Paarlberg, 2014). In naïve, pre-weaning piglets, PEDV has been shown to cause up to 90% mortality and PDCoV can cause between 40% to 90% mortality with 100% morbidity associated with both PEDV and PDCoV (Jung, Hu, & Saif, 2016; Madson et al., 2014; Stevenson et al., 2013). Today, both PEDV and PDCoV are endemic in U.S. swine herds.

SECoV are known to infect intestinal enterocytes causing atrophic enteritis leading to effusive diarrhea. Increased mortality of naïve neonate piglets resulted from rapid fluid loss and dehydration associated with SECoV infection. Age-dependent pathogenicity associated with PEDV occurs in the pre-weaning and nursery/growing stages of swine production. Due to a slower turnover of infected enterocytes and lack of immunological memory, neonates suffer more severe disease than weaned pigs. While the age-dependent mechanism remains unsolved, one study showed the evidence that impaired crypt stem cell regeneration and impaired lytic activity of infected enterocytes caused severe disease in 9-day-old pigs (Annamalai, Saif, Lu, & Jung, 2015; Jung, Eyerly, Annamalai, Lu, & Saif, 2015). In neonates, the intestinal barrier was disrupted causing shortening of villi and widening of intestinal crypts and reduced numbers of IFN-γ producing cytotoxic cells. In comparison, PEDV infection caused less severe lesions and

impact in 26-day-old pigs, resulting in reduced disease severity (Curry, Gabler, Schwartz, Yoon, & Burrough, 2016; Jung et al., 2015). In addition to age-related difference, there appears virus specific differences in the pathogenicity between PEDV and PDCoV as shown in our present work. PEDV infection caused high viral load in feces and tissues, as well as, longer detection of vRNA in tissues and feces when compared with PDCoV (Curry et al., 2017). Pathological lesions are severely diffuse throughout the small intestine during PEDV infection of 3-week-old pigs (Madson et al., 2014). PDCoV infection observed similar results in 2 to 3-week-old pigs, but the extent of viral detection was limited when compared with PEDV infection (Jung et al., 2016). These observed differences between the 2 viruses may be attributed to the ability of virus to replicate in older pigs. In conclusion, SECoV pathogenicity differences exist probably due to both host and virus factors.

The emergence of virulent and economically devastating SECoV encourages the development of enhanced biosecurity and vaccinations to prevent disease transmission. During the 2013 outbreak, more than 29 states across the U.S. had confirmed cases of PEDV (Chen et al., 2014). Today, the virus has been confirmed in most of the U.S. and across North America. While it is still unclear how PEDV entered the U.S., multiple lapses in biosecurity may have been attributed to its rapid spread across North America. From our current study, it was noticed that pigs can shed PEDV and PDCoV after the animals were no longer diarrheic. Fecal shedding of PEDV from subclinical animals has also been reported by other investigators (Madson et al., 2014; Niederwerder et al., 2016). As such, animals can contribute to the persistence and lateral transmission of the virus across the different stages of pig production and spread on other production systems, this observation should be taken into devising biosecurity measure including

managing transportation and vehicles (Lowe et al., 2014). Continuous monitoring for SECoV is critical particularly for grow-finishers and breeding animals.

The porcine gastrointestinal immune system of young pigs is slow to mature and develop, increasing the susceptibility to enteric viral disease when compared to an adult pig (Stokes, Bailey, & Haverson, 1991). The physical integrity of the gastrointestinal barrier is comprised of epithelial cells, mucus, and sIgA which form a protective barrier. When exposed to an enteric pathogen, gut-associated lymphoid tissue (GALT) stimulates the release of immune cells to assist in clearance of the pathogen. In sows exposed to TGEV virus, stimulated GALT increased both mucosal and cell-mediated immunity at the site of infection and migration to the mesenteric lymph node. When the same sows were given a homologous challenge dose post exposure, high levels of IgA B memory cells and T lymphocytes in the intestines prevented re-infection from occurring, conferring complete protection (Langel, Paim, Lager, & Vlasova, 2016; Saif, 1996). In a recent study, PEDV infected and recovered 8-week-old pigs demonstrated protection from homologous challenge. When challenged with PEDV, previously exposed pigs did not develop diarrhea or pathological lesions in the intestinal tissues, although 10% of pigs did have vRNA detectable in the feces. Moreover, increased levels of PEDV IgA were measured in the feces of challenged pigs which were shown to be protective from re-infection of homologous PEDV (Gerber et al., 2016). Understanding the mechanisms of immunological protection from SECoV infection in young pigs is essential to developing preventative measures that delivery sterilizing immunity.

Recommendations for Future Research

The underlying mechanisms of age-dependent and virus-dependent pathogenicity remains unsolved. Understanding SECoV pathogenicity comes mainly from studies done in neonate or pre-weaning pigs. Our study compared pathogenicity of PEDV and PDCoV infection in nursery animals and determined differences in the pathogenicity of SECoV in older animals. Both PEDV and PDCoV infected older pigs in this study, clinical manifestations of disease varied in growth and performance, mean viral load in tissues and feces, viremia, and serum antibody responses (Curry et al., 2017). The lack of information on the comparative pathogenesis weaned pigs warrants further investigation to understand the consequence of SECoV infection in older animals. Other limitations of this study include: use of tissue-culture adapted virus strains, unknown MID of PDCoV, lack of replication of observations, and unclear humoral immunity in PDCoV-infected pigs. Future studies should address these concerns. In conclusion, completing additional comparative pathogenicity studies in weaned pigs and comparing SECoV side-by-side in vivo can illuminate possible mechanisms, which can serve as targets for disease prevention.

A mechanism associated with gastrointestinal barrier disruption could explain the discrepancies between SECoV pathogenicity in different age pigs. I hypothesize, gastrointestinal barrier impairment caused by pro-apoptotic pathways as a possible mechanism for enhanced virulence and age-dependent pathogenicity in young age pigs infected with PEDV, and possibly other SECoV. In neonates, the gastrointestinal system is still immature and the mucosal immune system is developing. Phagocytic immune cells are important for maintenance of the mucosal immune system, specifically, autophagy influences intestinal T cell compartments by impacting antigen processing and lymphocyte homeostasis (Kabat, Pott, & Maloy, 2016). Additionally, TCR repertoires can change with age and antigen exposure. γδ-TCR is an important mucosal associated

receptor (Holtmeier et al., 2002). In young pigs, PEDV infection impairs the gastrointestinal barrier, more specifically it reduces the function of tight junctions, allowing the virus to cross the intestinal tissues. In PEDV infection of neonates, crypt cell proliferation declined, as pro-apoptotic pathways upregulated, causing severe pathogenicity (De Arriba, Carvajal, Pozo, & Rubio, 2002; Jung et al., 2015). PEDV infection of 6-week-old pigs reduced the expression of tight junctional proteins, resulting in impaired gastrointestinal barrier function (Curry et al., 2016). Therapies that target this pathway could prevent the inflammation and disruption of the gastrointestinal barrier. In Inflammatory Bowel Disease, disease drives changes in the homeostatic mechanisms of the mucosal immune system. Altering of these mucosal interactions may help mitigate the impact of IBD (Pandiyan & Lavelle, 2016). Antigen-presenting cells (APC) in the lamina propria and Peyer's Patch facilitate antigen uptake across the epithelium to induce antigen-specific immunity in presence of a gastrointestinal infection. Similarly, APC in the secondary lymphoid tissues, specifically the MLN, reside in the LN and sample antigen from lymphatic fluid, inducing an early T cell activation (Pandiyan & Lavelle, 2016). Due to the slow maturation of the cellular immune response in neonates, this could explain why pathogenicity is more severe than in older, more mature animals. Taken together, vaccines that induce effective mucosal immunity could provide protection from SECoV infection.

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